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(54) Title: CCR4 ANTAGONISTS IN SEPSIS

(57) Abstract

CCR4 receptor antagonists are used for treatment and/or prevention of sepsis and/or septic shock. The antagonists of this invention typically are selected among several classes but preferably are anti–CCR4 antibodies.

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CCR4 ANTAGONISTS IN SEPSIS

FIELD OF INVENTION

CCR4 receptor antagonists can be administered in therapeutically effective doses to treat and/or prevent sepsis and/or septic shock. The antagonists of this invention typically are selected among several classes but preferably are anti-CCR4 antibodies.

BACKGROUND OF INVENTION

Chemokines and their receptors are at the core of many processes in biology, from routine immunosurveillance and the inflammatory process, through to the infection of cells by HIV. In the past two years, various bioinformatic and cloning strategies have led to an explosion in the number of chemokines and receptors that have been identified. Although the picture is far from complete, several themes are emerging.

Chemokines (or chemoattractant cytokines) are a large family of small proteins that are involved both in the routine immunosurveillance that takes place in the body and in the activation and recruitment of specific cell populations during disease. Up until ten years ago, little was known about the proteins that might act as the traffic controllers to recruit specifically leukocyte subpopulations to sites of inflammation. The search for such factors led first to the identification of interleukin 8 (IL-8), a neutrophil chemoattractant, and monocyte chemotactic protein 1 (MCP-1), a monocyte and T-cell chemoattractant. Amino acid sequencing of these chemokines showed two different patterns of four conserved cysteine residues: in IL-8, the two N-terminal cysteines are separated by a single amino acid to form a CXC motif, whereas in MCP-1, they are adjacent, and form a CC motif. These spacings gave rise to the two principal chemokine subclasses, CXC (also known as -chemokines) and CC (also known as -chemokines). Although protein identity levels can be low as 20%, the three-dimensional structures of the monomeric proteins in both groups are almost superimposable.

The first two CXC chemokine receptors identified were found predominantly on neutrophils, and thus it became dogma that CXC chemokines were the drivers of acute

inflammation. Much work has focused on their role in diseases such as acute respiratory distress syndrome and septic shock (Folkesson H. G. et al. (1995), J. Clin. Invest., 96:107-116). By contrast, the CC chemokine receptors are expressed on a much wider range of cells, including lymphocytes, monocytes, macrophages, eosinophils, basophils and even platelets, and have been linked to chronic inflammatory diseases such as asthma, arthritis and atherosclerosis. This dichotomy has broken down because the most recently discovered CXC chemokine receptors CXCR3 and CXCR4 are expressed on T cells, and CXCR5 is expressed on B cells. In addition, it has long been known that murine neutrophils can express active CC chemokine receptors, and human neutrophils become responsive to CC chemokines following incubation with interferon (IFN-γ) (Bonecchi et al (1998).

The CC chemokine receptors CCR1-5 bind multiple CC chemokines. As a result of the new chemokines becoming available, the ligand range has also been extended for some receptors. For example, the novel CC chemokines Thymus and activation-related chemokine (TARC) and macrophage-derived chemokine (MDC) also bind to CCR4 (Imai et al., 1997; Imai et al., 1998), whereas CCR4 was initially described as being activated by macrophage inflammatory protein 1 (MIP-1), RANTES (regulated upon activation normal T expressed and secreted) and monocyte chemoattractant protein 1 (MCP-1) CXCR4, CCR6 and CX3CR1 remain highly selective, only binding to one chemokine out of 30 tested in most cases. It remains to be seen if this apparent selectivity holds out as new chemokines are discovered. The idea that restrictive expression of the receptor is associated with a restrictive ligand-binding pattern is attractive, but this might merely be an artifact of the recent discovery.

The role of chemokines in inflammation has also been validated by the use of monoclonal antibodies in inflammatory models: MIP-1 antibodies significantly reduce eosinophilia in the S. mansoni egg antigen model (Standiford T. J. et al. (1995) J. Immunol., 155:1515-1524); antibodies to IL-8 prevented neutrophil-mediated sepsis in the rabbit (Folkesson H. G. et al. (1995), J. Clin. Invest., 96:107-116; Yokoi K. I. et al. (1997) Lab. Invest.,

76:375-384); and anti-MCP-1 antibody significantly reduced cellular recruitment in glomerulonephritis (Lloyd C. M. et al. (1997) J. Exp. Med., 185:1371-1380) and granuloma models (Flory C. M. et al. (1993) Lab. Invest., 69:396-404). These results confirm that despite the apparent complexity of the system, elimination of a single chemokine or receptor can significantly alter models of pathology.

DESCRIPTION OF INVENTION

It has now been found that CCR4 plays a role in sepsis and that CCR4 receptor antagonists can be administered in therapeutically effective doses to treat and/or prevent sepsis and/or septic shock. The antagonists of this invention typically are selected among several classes but preferably are anti-CCR4 antibodies.

Therefore, the main object of the present invention is to provide a method to treat and/or prevent sepsis and/or septic shock in an individual comprising administering a therapeutically effective amount of CCR4 antagonist.

A still further object of the present invention is the use of a CCR4 antagonist together with a pharmaceutically acceptable carrier in the preparation of pharmaceutical compositions for treatment of sepsis and/or septic shock. The pharmaceutical compositions prepared in this way are also a further object of the present invention.

Sepsis is a serious infection caused by bacteria that has entered a wound or body tissue that leads to the formation of pus, or to the spread of the bacteria in the blood. Sepsis is a result of a bacterial infection that can originate anywhere in the body. Common sites are the genitourinary tract, the liver or biliary (liver secretion) tract, the gastrointestinal tract, and the lungs. Less common sites are intravenous lines, surgical wounds, surgical drains, and sites of skin breakdown known as decubitus ulcers or bedsores. The infection is usually confirmed by a positive blood culture. The infection can lead to shock, called septic shock. Low blood pressure and a change in mental status may be early-warning signs of shock.

There has recently been an increase in the occurance of sepsis caused by organisms that are resistant to most standard antibiotics. Therefore, new therapies for sepsis are highly desirable. Sepsis can be a life-threatening situation, especially in people with a weakened immune systems. The risk factors associated with sepsis include, for example, recent bacterial pneumonia, meningitis, a urinary tract infection that does not respond to antibiotics, osteomyelitis, bacterial peritonitis, a recent dental procedure, a recent endoscopy procedure, a recent cardiovascular procedure, an indwelling urinary catheter, a recent major surgery, cellulitis, a recent therapy with antibiotics, and the like. People whose immune systems are suppressed by therapies or by certain diseases are at higher risk for sepsis. The incidence of sepsis is 2 out of 10,000 people (see: http://health.yahoo.com/ health/Diseases_and_Conditions/Disease_Feed_Data/Sepsis/).

Septic shock is also called bacteremic shock. It is a serious, abnormal condition that occurs when blood flow through the body is insufficient resulting in low blood pressure and decreased urine output. It is caused by an overwhelming infection. Septic shock occurs more often in the very old and the very young and in people with other underlying illnesses. Many bacterial organisms can cause septic shock. Toxins released by bacteria can cause tissue damage and interfere with normal blood circulation. The risk factors include underlying illnesses such as diabethes; hematologic cancers, and diseases of the genitourinary system, liver or biliary system, and intestinal system. Other risk factors are recent infection, prolonged antibiotic therapy, and a recent surgery or procedure. The incidence is approximately 3 out of 1,000,000 people annually (see: http://health.yahoo.com/health/Diseases_and_Conditions/Disease_Feed_Data/Septic_shock).

The classical treatment of sepsis and sepsis shock is the immediate treatment with antibiotics as soon as sepsis or septic shock is suspected. Administration with antibiotics, usually intravenously, is necessary even before the causative infectious organism is identified by blood cultures. In view of the fact that more and more microorganisms have become resistant to antibiotics, especially microorganisms present in hospitals, a further

or additional possibility of treating sepsis and/or septic shock is highly desirable. The invention provides for such a treatment, namely using CCR4 antagonists. CCR4 antagonists can be preferably applied in addition to the treatment of the patient with antibiotics.

"Pharmaceutically acceptable" is meant to encompass any carrier, which does not interfere with the effectiveness of the biological activity of the active ingredient and that is not toxic to the host to which is administered. For example, for parenteral administration, the above active ingredients may be formulated in unit dosage form for injection in vehicles such as saline, dextrose solution, serum albumin and Ringer's solution. Besides the pharmaceutically acceptable carrier, the compositions of the invention can also comprise minor amounts of additives, such as stabilizers, excipients, buffers and preservatives.

The administration of such active ingredient may be by intravenous, intramuscular or subcutaneous route. Other routes of administration, which may establish the desired blood levels of the respective ingredients, are comprised by the present invention.

The active ingredients of the claimed compositions herein are CCR4 antagonists. Preferably, they are polypeptides that bind CCR4 with high affinity. The polypeptide typically binds to a specific epitope, thus blocking the receptor activity. In a highly preferred embodiment of the invention, the CCR4-antagonists are anti-CCR4 antibodies. CCR4 antibodies and fragments, variants or synthetic constructs of antibodies or derivatives of such molecules, as well as further CCR4-antagonists, can be prepared as specifically described in WO 96/23068.

As used herein, the term "antibodies" includes polyclonal antibodies, monoclonal antibodies, antigen-binding fragments hereof such as $F(ab')_2$ and Fab fragments, and the like, including genetically engineered antibodies. Specific antibodies typically bind to a CCR4 polypeptide with a K_d of greater than or equal to 10^7/M. The affinity of an

antibody can be readily determined by one of ordinary skill in the art (see, for example, Roit, *Essential Immunology, fifth Ed.* Blackwell Scientific Publications, 1984).

Methods for preparing polyclonal and monoclonal antibodies are well known in the art (see, for example, Sambrook et al., *Molecular Cloning: A Laboratory- Manual. second Ed.*. Cold Spring harbor, NY, 1989; and Hurrel, Ed., *Monoclonal Hybridoma Antibodies: Techniques and Applications.* CRC Press, Inc., Boca Raton, FL, 1982). As would be evident to one of ordinary skill in the art, polyclonal antibodies can be generated from a variety of warm-blooded animals such as horses, cows, goats, sheep, dogs, chickens, rabbits, mice, and rats. The immunogenicity of a peptide or polypeptide may be increased through the use of an adjuvant such as Freund's complete or incomplete adjuvant. A variety of assays known to those skilled in the art can be utilized to detect antibodies which specifically bind to polypeptides (see Harlow and Lane (Eds.), *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, 1988).

Monoclonal antibodies may be obtained by well-established methods, e.g. as described in A. Johnstone and R. Thorpe, *Immunochemistry in practice,* 2nd. Ed., Blackwell Scientific Publications, 1987, pp. 35-43.

Generally, monoclonal antibodies are produced by immunizing an animal with a biological specimen or other foreign substance, obtaining antibody-producing cells from the animal, and fusing the antibody-producing cells with strains of neoplastic cells, e.g. tumor cells, to produce hybridomas which are isolated and cultured as monoclones. The monoclonal hybridomas may either be cultured *in vitro* or may be grown *in vivo* as tumors in a host animal. Because each antibody-producing cell line produces a single unique antibody, the monoclonal cultures of hybridomas each produce a homogenous antibody population which may be obtained either from the culture medium of hybridoma cultures grown *in vitro* or from the ascitic fluid, or serum of a tumor-bearing host animal. Not all of the clones which result from fusion of neoplastic cells with antibody-producing cells are specific for the desired foreign substance or antigen, because many of the

hybridomas will secrete antibodies which the animal's immune system has generated in reaction to other foreign substances. Even monoclonal antibodies against the subject antigen will differ from clone to clone because antibodies produced by different clones may react with different antigenic determinants of the same molecule. From each clone, therefore, it is necessary to obtain the resulting antibody or the antibody-containing medium, serum or ascitic fluid and test its reactivity with the subject biological material and to test its specificity by determining what other biological material, if any, it recognizes.

When prepared by recombinant DNA techniques, the antibody may be produced by cloning a DNA sequence coding for the antibody or a fragment thereof into a suitable cell, e.g. a microbial, plant, animal or human cell, and culturing the cell under conditions conducive to the production of the antibody or fragment in question and recovering the antibody or fragment thereof from the culture. Possible strategies for the preparation of cloned antibodies are discussed in, for instance, L. Riechmann et al., 1988, describing the preparation of chimeric antibodies of rat variable regions and human constant regions; M. Better et al., 1988, describing the preparation of chimeric mouse-human Fab fragments; Sharra and Plückthun, 1988, describing the cloning of an immunoglobulin Fv fragment containing antigen-binding variable domains; and E.S. Ward et al, 1989, describing the cloning of isolated antigen-binding variable domains ("single-domain antibodies"). (Humanized monoclonal antibodies in general see, for example, *Molecular Biology and Biotechnology* (3rd Ed.), Walker and Gingold (Eds.), The Royal Society of Chemistry 1993, p 357-385).

Monoclonal antibodies or other genetically engineered antibodies with specificity for the CCR4 polypeptide are preferred as therapeutic agents for the treatment of sepsis and/or septic shock. The antibodies may then be humanized to reduce the immunogenicity. Humanization is done by grafting the Complementary-Determining Region (CDR) from the original murine antibody to the constant regions of a human antibody. Various

methods can he used to ensure the specificity and avidity of the grafted antibody (Queen et al, 1988).

Antibodies to CCR4 polypeptide may be used for isolation, for affinity purification, for diagnostic assays, for determination of circulating levels of CCR4 polypeptides, and as antagonists to block CCR4 activity *in vitro and in vivo*. (See, for example, *Immobilized Affinity Ligand Techniques*. Hermanson et al., eds., Academic Press, San Diego, CA, 1992, pp. 195-202).

CCR4 antagonist can be administered to an individual in a variety of ways. The routes of administration include intradermal, transdermal (e.g. in slow release formulations), intramuscular, intraperitoneal, intravenous, subcutaneous, oral, epidural, topical, and intranasal routes. Any other therapeutically efficacious route of administration can be used, for example absorption through epithelial or endothelial tissues or by gene therapy wherein a DNA molecule encoding the CCR4 antagonist is administered to the patient (e.g. via a vector) which causes the CCR4 antagonist to be expressed and secreted *in vivo*. In addition the CCR4 antagonist can be administered together with other components of biologically active agents such as pharmaceutically acceptable surfactants, excipients, carriers, diluents and vehicles.

For parenteral (e.g. intravenous, subcutaneous, intramuscular) administration, CCR4 antagonists can be formulated as a solution, suspension, emulsion or lyophilized powder in association with a pharmaceutically acceptable parenteral vehicle (e.g. water, saline, dextrose solution) and additives that maintain isotonicity (e.g. mannitol) or chemical stability (e.g. preservatives and buffers). The formulation is sterilized by commonly used techniques.

The therapeutically effective amounts of CCR4 antagonist will be a function of many variables, including the type of antagonist, the affinity of the antagonist for CCR4, any residual cytotoxic activity exhibited by competitive antagonists, the route of

administration, the clinical condition of the patient (including the desirability of maintaining a non-toxic level of endogenous CCR4 activity). A "therapeutically effective amount" is such that when administered, the CCR4 antagonist results in inhibition of the biological activity of CCR4. The dosage administered, as single or multiple doses, to an individual will vary depending upon a variety of factor, including CCR4 antagonist pharmacokinetic properties, the route of administration, patient conditions and characteristics (sex, age, body weight, health, size), extent of symptoms, concurrent treatments, frequency of treatment and the effect desired. Adjustment and manipulation of established dosage ranges are well within the ability of those skilled, as well as *in vitro* and *in vivo* methods of determining the inhibition of CCR4 in an individual.

Usually a daily dosage of active ingredient can be about 0.01 to 100 milligrams per kilogram of body weight. Ordinarily 1 to 100 milligrams per kilogram per day given in divided doses or in sustained release form is effective to obtain the desired results. Second or subsequent administrations can be performed at a dosage which is the same, less than or greater than the initial or previous dose administered to the individual. A second or subsequent administrations can be administered during or prior to relapse of the sepsis/septic shock or the related symptoms. The terms "relapse" or "reoccurrence" are defined to encompass the appearance of one or more of symptoms of sepsis or septic shock, respectively.

The present invention will now be illustrated by the following Examples, which are not intended to be limiting in any way, and makes reference to the following Figures.

DESCRIPTION OF THE FIGURES

Fig. 1a: Targeting strategy. Wild type CCR4 locus with partial restriction map (top), targeting vector (middle) and predicted structure of the targeted allele after homologous recombination (bottom). The coding region of the gene is shown as a black box. The neomycin resistance gene is dark grey and thymidine kinase gene is light grey. The arrows denote the position of the PCR primers used to

identify ES cell clones expressing the transgene. The probe used for screening genomic DNA is shown by the thick black bar (probe). Restriction sites: P, PstI; N, NheI; Ns, NsiI; A, AvrI; X, XhoI; E5, EcoRV; Ec, Eco47-3; H, HpaI; H3, HindIII.

Fig 1b: Representative Southern blot analysis of Pst1 digested tail DNA from wild type (+/+), heterozygote (+/-) and homozygous knockout mice CCR4 (-/-). The expected bands sizes of the wild type allele (4.5 kb) and the targeted allele (3.4kb) are indicated by arrows.

Fig 1c: RT-PCR analysis of chemokine receptor mRNA in spleen (lines 1 to 4) and thymus (lanes 5 to 8) of *CCR4*+/+ (lines 1, 2, 5, 6) and -/- mice (lanes 3, 4, 7, 8).

Fig. 1d: RT-PCR analysis of CCR4 mRNA in peritoneal marcrophages isolated from CCR4 +/+ mice (lanes 1 to 3) and CCR4 -/- mice (lanes 4 to 6). The predicted band size of CCR4 PCR product indicated by the arrow is 1.1 kb.

Fig. 1e: Chemotaxis of splenocytes in response to CCR4 ligands TARC, hMIP-α, RANTES, and MDC. Splenocytes isolated from *CCR4*+/+ are represented by circles, and -/- mice by squares. Results shown are the means of triplicate determinations for each concentration of chemokine, and are respresentative of at least four experiments.

Fig. 2a: Cytokine production by ConA stimulated naïve splenocytes from CCR4 -/- mice (white bars) and CCR4+/+ mice (hatched bars). Spleens from naïve mice were dispersed in culture (10^6 cells/ml) and stimulated with 5 μg/ml ConA. Cytokine levels were measured by ELISA 24h later.

Fig. 2b: Chemotactic response of in vitro derived TH2 T cells to MDC (top panel) and RANTES (lower panel). CCR4 +/+ (open symbols) and CCR4 -/- (closed panels).

Fig. 2c: CCR4 deficient mice develop allergic airways inflammation. Increased airway responsiveness to methacholine was measured by whole body plethysmography. This method allows measurements of spontaneous breathing in a non-anaesthetized mouse by recording respiratory pressure curves before and after

methacholine inhalation. From the curves, values for the enhanced pause (Penh) are calculated and used as an index of bronchial hyperresponsiveness (BHR). BHR in *CCR4+/+* mice (open symbols) and *CCR4-/-* (closed symbols) mice after priming with 10 µg of OVA in 0.2 ml of alum and followed by intra-nasal challenge with either 50 µl of 0.9 % NaCl (circles n=10) or OVA (0.3 mg/ml) (squares n=13).

Fig. 2d: Total cell count and individual leukocyte populations (eosinophils, macrophages, lymphocytes and neutrophils) in broncho-alveolar lavage (BAL) fluid. *CCR4+/+* (open bars n=13) and CCR4 (-/-) (hatched bars n=13) 72 h after the last OVA-challenge.

Fig. 2e: OVA-specific Ig titers in the sera of OVA-primed and challenged mice. *CCR4+/+* (open symbols) and *CCR4-/-* (closed symbols). Blood was sampled 72 h after the final intra-nasal OVA-challenge and was tested for the presence of anti-OVA IgM (squares), IgG1 (diamonds), IgG2a (circles) and IgE (triangles) by ELISA. Data is shown from one experiment representative of at least three different experiments for each parameter measured.

Fig. 3a: Survival curves of *CCR4+/+* and CCR4-/- mice injected intra-peritoneally with 60 mg/kg (squares, n=15), 90 mg/kg (circles, n=4) and 120 mg/kg (triangles, n=4) of LPS.

Fig. 3b: LPS induced thrombocytopenia after injection of 60 mg/kg LPS. CCR4 +/+, open squares (n=3); CCR4 -/-, filled squares (n=3).

Fig. 3c: Survival curves of CCR4 +/+ (open symbols) and CCR4 -/- (filled symbols) mice injected i.p. with 1 µg (squares, n=12), 2 µg (circles, n=4) and 4 µg (triangles, n=4) of LPS plus D-Gal (8 mg). The data shown is from 3 different experiments.

Fig. 3d: Bronchial hyperreactivity after intra-peritoneal injection of LPS. Results shown are means ± s.d. of 6 *CCR4+/+* and 8 *CCR4-/-* mice per group representative of at least two experiments.

Fig. 4a: Determination of serum TNF in *CCR4+/+* mice (filled squares) and *CCR4-/-* mice (open squares) after LPS treatment. On the x-axis, the time after LPS treatment is depicted, on the y-axis the amount of serum protein measured.

Cytokines were measured by ELISA using kits purchased from R&D systems. Results shown are the means ± s.d of 3 animals per group representative of at least 2 different experiments.

Fig. 4b: As Fig. 4a, but IL-1β measured.

Fig. 4c: As Fig. 4a, but IL-6 measured.

Fig 5a: Analysis of peritoneal lavage cells by cytospin. 8-12 week old mice were injected with LPS and killed by CO_2 asphyxiation 0, 1.5 , 3 and 24 h later. Peritoneal cells were recovered by lavage with 0.9% NaCl, counted, and 5 x 10^4 cells were cytospun and stained with Diff-Quik. Fig. 5a shows a micrograph of the cells at 0 h after (i.e. before) LPS in CCR4 -/- cells.

Fig. 5b: As Fig. 5a, but in CCR4 +/+ cells.

Fig. 5c: As Fig. 5a, but 1,5 h after LPS injection.

Fig. 5d: As Fig. 5b, but 1,5 h after LPS injection.

Fig. 5e: As Fig. 5a, but 3 h after LPS injection.

Fig. 5f: As Fig. 5b, but 3 h after LPS injection.

Fig. 5g: As Fig. 5a, but 24 h after LPS injection.

Fig. 5h: As Fig. 5b, but 24 h after LPS injection.

Fig. 6a: FACs analysis of peritoneal lavage cells after LPS treatment. Lavage cells were harvested as described in Fig. 5 and resuspended at 10^6/ml in FACs buffer. Cells were first incubated with Fc block (Pharmingen) for 10 min at 4 C, washed twice with FACs buffer and then incubated for 20 min with with PE labelled GR1 (Pharmingen). GR1 antigen is a marker for granulocytes. Cells were washed twice, resuspended in 200 µl FACS buffer and analyzed on a Becton Dickinson FACstar. Fig. 6a shows the percentage of GR1 positive cells (neutrophils) after 0 (i.e. before LPS treatment), 1,5 or 3 or 24 hours after LPS treatment.

Fig. 6b: As Fig. 6a, but using FITC labelled F4/80 (Serotec), T4/80 being a macrophage marker.

Fig. 6c: FACs result of F4/80 positive cells at time 0 hours after (i.e. before) LPS injection. Thick line represents CCR4 +/+, and thin line CCR4-/- mice.

Fig. 6d: As Fig. 6c, but analysis of 24 hours after LPS injection.

Fig. 7a. Time course of LPS induced cytokines. Serum levels of TNFα after 60 mg/kg LPS treatment.

Fig. 7b: *In vitro* production of TNFα by peritoneal lavage cells stimulated with 1 μg/ml LPS, $CCR4^{+/+}$ mice (open bars, n=3) and $CCR4^{-/-}$ mice (hatched bars, n=3). Results shown are representative of at least 2 different experiments.

Fig. 7c: As Fig. 7a, but serum levels of IL-1β shown .

Fig. 7d: As Fig. 7a, but serum levels of MIP-1α shown.

Fig. 8a: Analysis of peritoneal lavage neutrophils on cytospin slides prepared from the peritoneal lavage at various times (x-axis) after high dose LPS treatment indicated at x-axis of the figure.

Fig. 8b: As Fig. 8a, but macrophages analyzed.

Fig. 8c: The effect of LPS treatment (various times, x-axis) on marcrophage chemokine expression by peritoneal cells as measured by semi-quantitative PT-PCR. Fig. 8c shows MIP-2expression on CCR4 +/+ mice (open bars, n=3) and CCR4 -/- (hatched bars, n=3).

Fig. 8d: As Fig. 8c, but mMDC chemokine analyzed.

EXAMPLES

Example 1: Generation of CCR4 Knockout mice

Methods

1. Generation of CCR4 deficient mice

The murine CCR4 gene was isolated from an HM-1 embryonic stem cell library in λFIXII vector (Stratagene) by plaque hybridisation using murine CCR4 cDNA as a probe (Hoogewerf A.J. et al., (1996), Biochem. Biophys. Res. Commun. 218, 337-343). Two unique clones of 12.18 kb and 13.08 kb were shown to contain the mCCR4 coding sequence by PCR, using specific primers. An 8.5 kb fragment of genomic DNA identified to contain the CCR4 coding sequence by Southern blotting was subcloned into

pBluescript II SK- to generate pCCR4. The entire CCR4 coding sequence was then removed as an NheI/HpaI fragment and replaced with a neo cassette (derived from plasmid pMC1neoPolyA (Clontech). The resultant construct was digested EcoRV and Eco47-3, and religated, to generate a plasmid containing a long arm of homology of 4904 bp and a short arm of homology of 1318 bp. Finally, a thymidine kinase (tk) cassette was inserted into the HindIII/XhoI site of the plasmid to produce the targeting vector. The targeting vector was linearized with NotI and electroporated into HM-1 embryonic stem cells as described previously (Conquet F. et al., (1994), Nature 372, 237-243). Gancyclovir and G418 resistant clones were selected. DNA was isolated from resistant clones using DNAzol (Gibco-BRL), and the presence of the transgene was detected by PCR, and verified by Southern hybridization following PstI digestion of genomic DNA using a 466 bp probe derived by AvrII/NsiI digestion of pCCR4. Seven independent transgene-containing ES cells were used to produce chimeric mice by blastocyst injection according to standard procedures (McMahon A.P and Bradley A. (1990). Two 100% chimeric females were mated with a 100% chimeric male to generate heterozygous CCR4 (+/-) mice, and littermates from the matings of heterozygous mice were analyzed for the presence of homozygous CCR4 (-/-) knockout mice by southern blot analysis or by PCR on tail DNA.

Chemotaxis assays were performed using the micro Boyden chamber method. Recombinant human and mouse chemokines were purchased from R&D systems.

2. Reverse transcriptase PCR (RT-PCR)

Total RNA was isolated from thymocytes, splenocytes or peritoneal lavage cells using Trizol™ (Gibco BRL). One µg of total RNA from thymocytes and splenocytes or total RNA from 1 x 10^6 peritoneal exudate cells was reverse transcribed using Superscript™ (Gibco BRL) and oligo dT_{12-18} primer according to the manufacturer's instructions. One twentieth of the cDNA synthesis reaction was then subjected to 25 cycles of PCR using AmpliTaq™ (Perkin-Elmer, Norwood, CA) and PCR primers based on the Genbank/EMBL database entries for MIP-2 (X53798), mMDC (AF052505) and mCCR4 (X90862). PCR products were analyzed on 1 % agarose gels stained with ethidium

bromide, and bands migrating at the correct molecular weight were verified by direct sequencing. For semi-quantitative RT-PCR, bands were quantitated using Kodak Digital Science version 1.0 software and results are expressed as arbitrary units of mRNA.

3. In vitro differentiation of TH1 and TH2 cells

$CD8^+$ and Ig^+ depleted cells from lymph nodes and spleens of naïve $CCR4^{+/+}$ and $CCR4^{-/-}$ mice were cultured for 4 days on plates coated with an anti-CD3 antibody (145-2C11, Pharmingen, San Diego, CA) as previously described (16) in the presence of either murine IL-12 (500 pg/ml, R&D Systems, Abingdon, UK) plus anti-IL-4 monoclonal antibody (10 μg/ml) or murine IL-4 (500 U/ml, ImmunoKontact, Frankfurt, Germany) plus anti-IFN-γ monoclonal antibody (10 μg/ml, Pharmingen). After a 5 day culture period, cells were washed and re-stimulated for 24 h at a density of 2×10^5 cells per well on an anti-CD3-coated 96-well plate (Costar) in the presence of murine IL-2 (50 U/ml, R&D Systems).

4. Animals and treatments

Homozygous $CCR4^{-/-}$ mice were back crossed with C57BL/6 mice (Centre d'Elevage Janvier, Le Genest Saint-Isle, France) for 4 generations. Age and weight-matched $CCR4^{-/-}$ and $CCR4^{+/+}$ littermates from heterozygote ($CCR4^{+/-}$) matings from the fourth back cross were used in this study to control for strain background. Mice (20-25g) of either sex were immunized intraperitoneally (i.p.) with 10 μg of ovalbumin (OVA; A-5503, Sigma Chemical Corp., St Louis, MO) in 0.2 ml of alum (Serva, Heidelberg, Germany). Control mice received an injection of saline (0.9% w/v NaCl) alone. Fourteen days later, mice were anaesthetized by inhaled 2% FORENE ™ (Abbott, Cham, Switzerland) and 50 μg of OVA was administered to the lungs (in 50 μl of saline) intranasally as described previously (17). Control mice received 50 μl saline only. This procedure was repeated daily for 5 days. Tsuyuki et al., 1997). Animals were finally killed by lethal injection of 60 mg/kg pentobarbitone. Statistical analysis was using Student's T test except, for the analysis of the survival curves the Log-Rank test (two tailed) was performed.

Phenol extracted bacterial lipopolysaccharide (LPS) from Escherichia coli 055:B5 (List Biological Laboratories, Inc. Campbell, CA) was administered at 60, 90, and 120 mg/kg i.p. for the high-dose LPS shock model. For the low dose LPS shock model, mice received 1, 2, and 4 µg of LPS with 8 mg D-galactosamine (D-Gal) (Fluka, Buchs, Switzerland) in 0.5 ml saline. Animals were killed by CO_2 asphyxiation at the time points indicated in the figures.

5. Chemotaxis assays

Spleens from $CCR4^{+/+}$ and $CCR4^{-/-}$ mice were dispersed through a 70 µm nylon cell strainer (Becton Dickinson). Erythrocytes were removed by hypotonic lysis. Cells were harvested by centifugation and resuspended in RPMI medium containing 5 % FCS and 2 mM glutamine. Chemotaxis assays were performed using the micro-Boyden chamber method with 5 µm filters (Lusti-Narasimhan,et al., 1996). Recombinant human and mouse chemokines were purchased from R&D Systems or made in house.

Results

The murine CCR4 gene was deleted through homologous recombination using the targeting vector shown in Fig. 1a. Targeted ES cells were used to generate chimeric mice which transmitted the transgene through the germ line. Southern blot analysis confirmed that the CCR4 gene had been targeted (Fig. 1b). CCR4 knockout mice were viable, appeared to develop normally, and showed no overt morphological or behavioural defects in the unstressed state. Normally, CCR4 mRNA is expressed in T cells, predominantly in the thymus, spleen and peripheral blood (Power C.A. et al., (1995), J. Biol. Chem. 270, 19495-19500; Hoogewerf A.J. et al., (1996), Biochem. Biophys. Res. Commun. 218, 337-343). Reverse transcriptase(RT)-PCR was used to demonstrate that the mRNA for CCR4 was not present in the spleen and thymus of the targeted animals (Figure 1c). Further, it was demonstrated that peritoneal macrophages in CCR4 deficient mice do not express CCR4 mRNA (Fig. 1d).

Although originally identified as a receptor for MIP-1α and RANTES, CCR4 is in fact a high affinity receptor for two recently described chemokines, thymus and activation

regulated chemokine (TARC) (Imai T. et al., (1997), J. Biol. Chem. 272, 15036-15042) and macrophage derived chemokine (MDC) (Imai T., et al. (1998), J. Biol. Chem. 273, 1764-1768). Therefore, the ability of splenocytes and thymocytes isolated from the targeted and wild type mice to migrate in response to the proposed CCR4 ligands was analyzed. Splenocytes from *CCR4-/-* mice had no chemotactic response to TARC or MDC whereas splenocytes from *CCR4+/+* mice responded with the characteristic dose-response curve (Fig. 1e), confirming that the gene deleted in this study is an endogenous TARC and MDC receptor. However, splenocytes isolated from the CCR4-/- mice responded neither to human MIP-1α nor to murine MIP-1α (data not shown). This was surprising since the response to RANTES was similar in both the CCR4+/+ and -/- mice. All of the RANTES receptors described to date (CCR1, CCR5, and CCR3 in mouse) have also been shown to bind and signal in response to MIP-1α *in vitro*. RT-PCR analysis of the cell populations used in the study confirmed that deletion of the CCR4 gene did not interfere with expression of these receptors at the mRNA level (data not shown). Taken together these results indicate that under these conditions, CCR4 is a physiological receptor for MIP-1α.

Example 2: CCR4 deletion in a Th2 type disease

Methods

1. Evaluation of bronchial hyperresponsiveness

Bronchial hyperresponsiveness (BHR) was measured by recording respiratory pressure curves by whole body plethysmography (Hamelmann et al., 1997) in response to inhaled methacholine (MCh) (Aldrich-Chemie, Steinhein, Germany) using a Buxco® apparatus (EMKA Technologies, Paris, France). The airway reactivity was expressed in enhanced pause (Penh) as described previously.

2. Analysis of blood and lavage fluids

Blood (20 µl) was collected from the retro-orbital plexus of mice into a heparinized micropipette then transferred to an Unopette micro collection system for platelet

determination (Becton Dickinson, Rutherford, NJ). Three independent samples of platelets were counted in a haemocytometer. A minimum of 100 cells were counted, and the arithmetic mean of the three counts was calculated (Tacchini-Cottier et al., 1998).

OVA-specific IgM, IgG1, IgG2a and IgE titers were measured in serum samples obtained three days after the final intranasal treatment with OVA using a standard ELISA protocol (19). Quantification of murine TNFα in serum and in culture supernatant, and IL-1β, IL-6 and MIP-1α in serum was determined using cytokine-specific ELISAs as per the manufacturer's protocol (R&D Systems).

BAL and peritoneal lavage cells were harvested and differential cell counts were performed on cytospin preparations stained with Diff-Quick(TM) (Baxter Diagnostics, Düdingen, Switzerland). A minimum of two hundred cells were counted per field, with 3 fields per sample for BAL, and 5 fields per sample for peritoneal lavage.

For phenotypic analysis of peritoneal cells by flow cytometry, lavaged cells were resuspended at 10^6 cells/ml in PBS containing 1% BSA and 0.01% azide (FACS buffer). Cells were incubated with Fc block (Pharmingen) for 10 min at 4 C, washed twice with FACS buffer then incubated for 20 min with FITC-labeled rat anti-mouse F4/80 antibody (Serotec, Oxford, UK). Cells were washed twice, resuspended in 200 µl FACS buffer and analysed on a Becton Dickinson FACScan® flow cytometer with CellQuest™ software.

Results

Increasing numbers of reports show that CCR4 is highly expressed in human Th2 polarized cells (Sallusto F. et al, (1998), J. Exp. Med. 187, 875-883; Bonecchi R. et al, (1998) J. Exp. Med. 187, 129-134; D'Ambrosio D. et al., 1998), which are also responsive to the CCR4 ligands TARC and MDC, suggesting a possible role for this receptor in the development of Th2 responses. However at present, it is unclear if this paradigm can be applied to murine T cells.

Therefore, the ability of naïve splenocytes from wild type and CCR4 knockout mice to produce Th1 and Th2 cytokines in response to concanavalin A (ConA), a potent polyclonal activator of T cells, was assessed. Splenocytes from *CCR4-/-* produced

comparable levels of IL-2 and IFN-γ to *CCR4+/+* mice and slightly elevated levels of the Th2 cytokine IL-4, indicating that the cells from *CCR4-/-* mice were not generally defective in the production of these cytokines (Fig. 2a).

The role of CCR4 in the *in vitro* differentiation of T cells was addressed next. Naïve $CD4^+$ T cells were cultured for 4 days on plates coated with an anti-CD3 antibody either in the presence of IL-12 plus anti-IL-4 antibody, which induces Th1 cell differentiation, or in the presence of IL-4 plus anti-IFN-γ antibody, which induces Th2 cell differentiation. Cells were then re-stimulated with the anti-CD3 antibody for 24 h. $CD4^+$ T cells initially cultured in the presence of IL-4 showed no significant difference in the production of IL-4 ($CCR4^{+/+}$ T cells: 105 ± 15 pg/ml and $CCR4^{-/-}$ T cells: 190 ± 10 pg/ml) after re-stimulation. Likewise, T cells cultured with IL-12 produced comparable levels of IFN-γ ($CCR4^{+/+}$: 76 ± 19 ng/ml and $CCR4^{-/-}$: 49 ± 8 ng/ml). These results further show that *in vitro*, Th2 and Th1 cell differentiation was not impaired in the $CCR4^{-/-}$ mice.

As *in vitro* derived Th2 T cells have previously been shown to express CCR4, it was tested whether the cells used in this study could respond to CCR4 ligands in chemotaxis assays. Cells derived from the $CCR4^{-/-}$ mice failed to migrate in response to MDC whereas cells derived from the $CCR4^{+/+}$ mice had a robust chemotactic response to MDC (Figure 2b). A similar result was obtained using TARC (data not shown). In addition, *in vitro* derived Th2 T cells from both wild type and $CCR4^{-/-}$ mice responded to RANTES (Figure 2b).

Then, the effect of the CCR4 deletion in an ovalbumin-induced murine model of airways inflammation, a predominantly Th2 type disease, was studied.

Repeated intranasal ovalbumin (OVA) challenges in immunised *CCR4-/-* and +/+ littermates resulted in a significant increase in bronchial hyperreactivity to inhaled methacholine (Hamelmann E. et al., (1997), Am. J. Respir. Crit. Care Med. 156, 766-775) in both groups of animals when compared to saline-challenged mice (Fig. 2c). Penh

values were 0.8 ± 0.10 and 1.77 ± 0.2 in saline and OVA-challenged *CCR4+/+* mice respectively and 0.64 ± 0.11 and 1.88 ± 0.33 in saline-and OVA-challenged *CCR4-/-* mice respectively. Comparable ovalbumin-induced eosinophilia was observed in *CCR4+/+* and -/- littermates (Fig.2d), a finding consistent with the selective induction of a Th2 response in the airways (Chvatchko Y. et al, (1996), J. Exp. Med. 184, 2353-2360). No significant differences were observed in the broncho-alveolar lavage fluid in either the total cell count, or in other leukocyte populations (macrophages, lymphocytes and neutrophils) between OVA-challenged *CCR4+/+* and -/- littermates (Fig. 2d).

To confirm that efficient antigen priming had occurred in the periphery, serum titers of OVA-specific IgM, IgG1 and IgE in OVA-sensitised and challenged *CCR4+/+* and -/- littermates were analyzed, see Fig. 2e (Blyth D.I. et al., 1996) Am. J. Resp. Cell Mol. Biol. 14, 425-438). Again, OVA-specific IgM, IgG1 and IgE titers were comparable in *CCR4+/+* and -/- littermates (Fig. 2e). Taken together, these results suggest that deletion of the CCR4 gene does not impair the development of a Th2 response *in vivo*.

Example 3: CCR4 knockout mice in LPS-induced septic shock

The methods used in this example can be taken from examples 1 and 2.

CCR4 is also expressed on other cell types such as platelets (Power et al., 1995), monocytes (Power et al., 1995) and macrophages (Parums, D and Power, C.A., unpublished data and also Figure 1d).

Therefore the effect of the CCR4 deletion during LPS induced endotoxic shock, an inflammatory model in which these cells types have been implicated (Pajhrt et al., 1996 and Freudenberg et al., 1986), was assessed. LPS (60 – 120 mg/kg) was injected i.p. into $CCR4^{+/+}$ and $CCR4^{-/-}$ littermates and survival was assessed daily for 6 days (Figure 3a). All $CCR4^{+/+}$ mice died between 2 and 4 days after LPS injection. In contrast, 14 out of 15 $CCR4^{-/-}$ mice were alive on day 6, demonstrating significant resistance to 60 mg/kg LPS ($P < 0.001$). Indeed, the $CCR4^{-/-}$ mice were also strikingly resistant to LPS doses of up to 120 mg/kg. Control mice (saline injected $CCR4^{-/-}$ mice, n=4, and $CCR4^{+/+}$ mice, n=4),

remained alive and healthy throughout the 6 day study (data not shown). Interestingly, during the first few hours after LPS administration, $CCR4^{-/-}$ mice still showed signs of endotoxaemia such as shivering and lethargy. However, these effects were visually milder than in the $CCR4^{+/+}$ mice.

Intraperitoneal injection of a high dose of LPS is followed by a marked thrombocytopenia and accumulation of platelets in the liver and spleen (Shibazaki et al, 1996). Blood samples from $CCR4^{-/-}$ and $CCR4^{+/+}$ mice contained similar numbers of platelets. Furthermore, in an independent experiment, a super-imposable decrease in blood platelet count occurred in both $CCR4^{+/+}$ (n=3) and $CCR4^{-/-}$ mice (n=3) in the first 20 h after injection of high dose LPS (Figure 3b), indicating that there was no obvious difference in platelet mobilization between the two groups. However the platelet count returned to normal in the $CCR4^{-/-}$ mice by 5 days after treatment.

As a comparison, the effect of the CCR4 deletion in a low dose LPS endotoxic shock model was studied. In this model, the susceptibility of mice to a low doses of LPS (1, 2 and 4 μg) is enhanced by co-injection of 8 mg of D-galactosamine (D-gal) (Galanos et al., 1979). Within 24 h of i.p. injection of the LPS and D-gal combination, only 1 in 12 of the $CCR4^{+/+}$ mice survived whereas 9 out of 12 of the $CCR4^{-/-}$ littermates survived when treated with 1 μg of LPS plus D gal ($P < 0.02$) (Figure 3c), also demonstrating increased resistance to low-dose LPS. However when administered at 4 μg of LPS with D-gal, all $CCR4^{-/-}$ mice tested died but with a 6 h delay compared to the wild type mice.

The presence of bacterial products such as LPS in the blood stream is circulatory collapse and severe hypotension, which are associated with potentially lethal conditions including acute lung injury. LPS administration leads to an increase in the vascular permeability in the lungs (Standiford T.J.et al., (1995) J.Immunol. 155, 1515-1524). As a consequence of this, mice develop bronchial hyperreactivity (Y. Chvatchko et al., manuscript in preparation). Bronchial hyperreactivity was observed only in the *CCR4+/+* mice with

peaks at 9 h and 18 h after LPS injection, (Fig. 3d). *CCR4-/-* mice had little or no bronchial hyperreactivity.

LPS stimulates the release of inflammatory cytokines such as TNFα and IL-1β from monocyte/macrophages and neutrophils. It was shown that the LPS-induced pulmonary response is preceded by the production of TNFα. Interestingly, *CCR4-/-* mice failed to release significant levels of TNFα in response to LPS injection compared to *CCR4+/+* mice (Fig. 4a). This suggests that the observed resistance to LPS may be in part due to decreased TNFα implying that CCR4 may be indirectly involved in the regulation of TNFα. In addition to TNFα, a decreased IL-1β in serum was also observed (Fig 4b). The production of IL-6 (mainly by hepatocytes) occurs after gram-negative bacterial infection or TNFα infusion (Akira S. et al., (1993), Adv. Immunol. 54, 1-78). No difference in IL-6 production between *CCR4+/+* and -/- mice (Fig. 4c) was observed, suggesting that in the *CCR4-/-* mice the regulation of TNFα and IL-1β is independent from that of IL-6.

Then, the cellular composition of the peritoneal lavage at various times after LPS injection by cytospin analysis was looked at. No major differences were seen in the numbers and types of leukocytes recruited at early time points (Figs. 5 a-f). However, 24 h after LPS treatment, the leukocyte population in the peritoneal lavage of *CCR4-/-* mice was almost entirely composed of neutrophils (>80%) with few macrophages and no lymphocytes detectable (Fig. 5g) whereas in *CCR4+/+* mice, macrophages still comprised nearly 70% of the lavage cells, although the number of neutrophils was significantly increased from that seen at earlier time points (Fig. 5h). The enhanced neutrophil recruitment seen in the *CCR4-/-* mice may be in response to a defect in macrophage recruitment (see below). At 1.5 h and 3 h after LPS treatment, lavage fluid from *CCR4+/+* also contained large numbers of erythrocytes (Figs. 5d, f) which may be indicative of increased vascular permeability or haemorrhaging in these mice. Erythrocytes were absent or markedly reduced in the lavage of *CCR4-/-* mice (Figs. 5c,e).

Elevated TNFα production in the *CCR4+/+* mice may be associated with the increased vascular permeability. It is also possible that the absence of haemorrhaging observed in the *CCR4-/-* mice results from altered platelet function, although at present there is no experimental evidence in support of this hypothesis.

Next, the expression of the macrophage (F4/80) and granulocyte (GR1) markers in the peritoneal lavage cells was analyzed by FACs. Few GR1 positive staining cells were initially detected in the lavage but gradually increased in both *CCR4+/+* and -/- mice up to 24 h after LPS treatment (Fig. 6a). At this time there was a striking difference in the number of GR1 positive cells present in the CCR4-/- mice compared to *CCR4+/+* mice. In contrast, F4/80 positive cells (Fig. 6b) decreased in both groups of mice with time after LPS treatment. Interestingly in the *CCR4-/-* mice at 24 h there was not only a marked reduction in the total number of F4/80 expressing cells but there was also a decrease in the F4/80 expression level (Figs. 6c,d). These results imply a defect or deficiency in a particular macrophage population expressing F4/80. The F4/80 antigen is an unusual seven transmembrane receptor in that its extracellular domain is composed of EGF domain repeats. Its ligand(s) and function remain unknown but our results suggest that it may be important in the mechanism of LPS resistance in *CCR4-/-* mice.

LPS is known to stimulate the release of pro-inflammatory cytokines such as TNFα and IL-1β from monocytes, macrophages and neutrophils (Gutierrez-Ramos et al., 1997). Therefore the effect of the high dose LPS response was studied in more detail. A sharp increase in serum TNFα levels was observed in $CCR4^{+/+}$ mice 1.5 h after LPS injection, which returned to baseline by 4 h after treatment. Interestingly, $CCR4^{-/-}$ mice failed to induce significant levels of serum TNFα after LPS injection ($P<0.002$ at 1.5 h) (Figure 7a). Cells isolated by peritoneal lavage from naive $CCR4^{-/-}$ mice produced significantly lower levels of TNFα when cultured for 18 h in the presence of LPS, compared to cells isolated from naive $CCR4^{+/+}$ mice (107 ± 11 pg/ml compared to 201 ± 18 pg/ml, $P<0.0079$) (Figure 7b). In addition, a six fold decrease in serum IL-1β in $CCR4^{-/-}$ mice was observed

3 h after LPS injection ($P<0.002$), when compared to $CCR4^{+/+}$ mice (Figure 7c). This suggests that the observed resistance to LPS may in part be due to decreased TNFα and IL-1β production, implying that CCR4 is indirectly involved in the production of these cytokines. In contrast, IL-6 production was unaltered (data not shown), suggesting that in $CCR4^{-/-}$ mice, the regulation of IL-6 can be independent from that of TNFα and IL-1β. Further analysis of the $CCR4^{-/-}$ samples also revealed a parallel decrease in serum MIP-1α levels ($P \leq 0.0249$ at 1.5h) (Figure 7d) pointing to a possible macrophage defect.

The cellular composition of the peritoneal lavage was therefore assessed at various times after injection of high-dose LPS (Fig. 8a, b, c, d, x-axis) ere seen in the total number of cells at early time points. At 24 h after high-dose LPS treatment, the number of neutrophils detected in the lavage of both groups of mice was comparable (Figure 8a). This is as expected as to date there is no evidence for the expression of CCR4 on neutrophils. Furthermore, it was shown that neutrophils isolated from the peritoneal cavity of wild type mice after thioglycollate treatment do not respond to CCR4 ligands in chemotaxis assays. In addition thioglycollate elicited neutrophils from $CCR4^{-/-}$ mice respond normally to MIP-1α and MIP-2 (unpublished data). However $CCR4^{-/-}$ mice had significantly fewer macrophages than $CCR4^{+/+}$ mice ($P < 0.0099$) (Figure 8b). These findings were consistent with the observed decrease in the mRNA expression of the macrophage associated chemokines, macrophage inflammatory protein–2 (MIP-2) (Walley et al., 1997; Godiska et al., 1997), see Figures 8c and d. The apparent differences in macrophage numbers seen on the cytospins by FACs analysis for macrophage markers were thus confirmed.

Resistance to LPS induced lethality has now been demonstrated in a number of gene targeted mice including MIF (macrophage migration inhibition factor) -/- mice (Rodenburg et al., 1998). A possible mechanism of LPS resistance in the CCR4 deleted mice may be due to down regulation of MIF production by peritoneal macrophages.

However there was no evidence for this at the level of MIF mRNA as measured by RT-PCR (data not shown).

Whilst a great deal is known about the intracellular events that occur following LPS treatment, relatively little is known about the actual events which occur at the cell surface and the signal transduction mechanism by which LPS induces host cell activation. In the currently accepted model, LPS monomers are catalytically transferred by a lipid exchange molecule LBP (Bozza et al., 1999), to CD14, a major LPS receptor that lacks a transmembrane domain. The LPS transmembrane coreceptor toll receptor like 4 (Tlr4), is then responsible for the initiation of cellular responses after interaction with the LPS-CD14 complex. The roll of Tlr4 in LPS signalling has been demonstrated following the identification of strains of mice which contained mutations in the Tlr 4 gene rendering them resistant to the lethal effects of LPS. There were no differences in expression of Tlr 4 mRNA in *CCR4-/-* mice compared to *CCR4+/+* mice in peritoneal lavage cells (data not shown) and LPS resistance was not due to the presence of a mutated Tlr 4 coding sequence in the *CCR4-/-* mice..

Identification of the precise mechanisms of LPS induced cell stimulation is important for our understanding of bacterial pathogenesis and for the development of strategies to protect against gram negative bacterial infection. Targeted deletion of the CCR4 gene reveals an unexpected role for this receptor in LPS induced sepsis.

In conclusion, it was shown that 24 h after high dose LPS treatment there is a significant reduction in the numbers of macrophages found in the peritoneum of $CCR4^{-/-}$ mice. At the same time a decrease in macrophage associated serum cytokines TNFα, IL-1β and MIP-1α was noted. In addition, peritoneal lavage cells had decreased levels of mRNA for the chemokines MDC and MIP-2, which are thought to be produced mainly by activated macrophages. Taken together, these results are consistent with either a defect in macrophage function or the absence of a specific population, which is supported by the disappearance of the $F4/80^{+}$ population at 24 h after LPS treatment. Identification of the precise mechanisms of LPS induced cell stimulation is important for our understanding of

bacterial pathogenesis and for the development of strategies to protect against gram negative bacterial infection.

Targeted deletion of the CCR4 gene has revealed an unexpected role for this receptor in LPS induced endotoxic shock. Therefore, neutralization of CCR4 either by antibodies or chemokine receptor antagonists are a new therapeutic approach for the treatment of sepsis and/or septic shock.

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CLAIMS

1. Use of a CCR4 antagonist together with a pharmaceutically acceptable carrier in the preparation of a pharmaceutical composition for the treatment and/or prevention of sepsis.

2. Use of a CCR4 antagonist together with a pharmaceutically acceptable carrier in the preparation of a pharmaceutical composition for the treatment and/or prevention of septic shock.

3. The use of claim 1 or 2, wherein said CCR4 antagonist is a polypeptide able to bind CCR4.

4. The use of any of claims 1 to 3, wherein the CCR4 antagonist is an anti–CCR4 antibody or a fragment thereof.

5. The use of claim 4 wherein the monoclonal antibody is selected from the group consisting of: a chimeric monoclonal antibody, a humanized monoclonal antibody or fragment thereof.

6. A method for treating and/or preventing sepsis which comprises administering to a patient a therapeutically effective dose of a CCR4 antagonist.

7. A method for treating and/or preventing septic shock which comprises administering to a patient a therapeutically effective dose of a CCR4 antagonist.

8. Pharmaceutical composition comprising a CCR4 antagonist, together with a pharmaceutically acceptable carrier, in the treatment and/or prevention of sepsis.

9. Pharmaceutical composition comprising a CCR4 antagonist, together with a pharmaceutically acceptable carrier, in the treatment and/or prevention of septic shock.

10. The pharmaceutical composition according to claim 8 or 9, wherein the CCR4 antagonist has the features set out in any one of Claims 1 to 5.

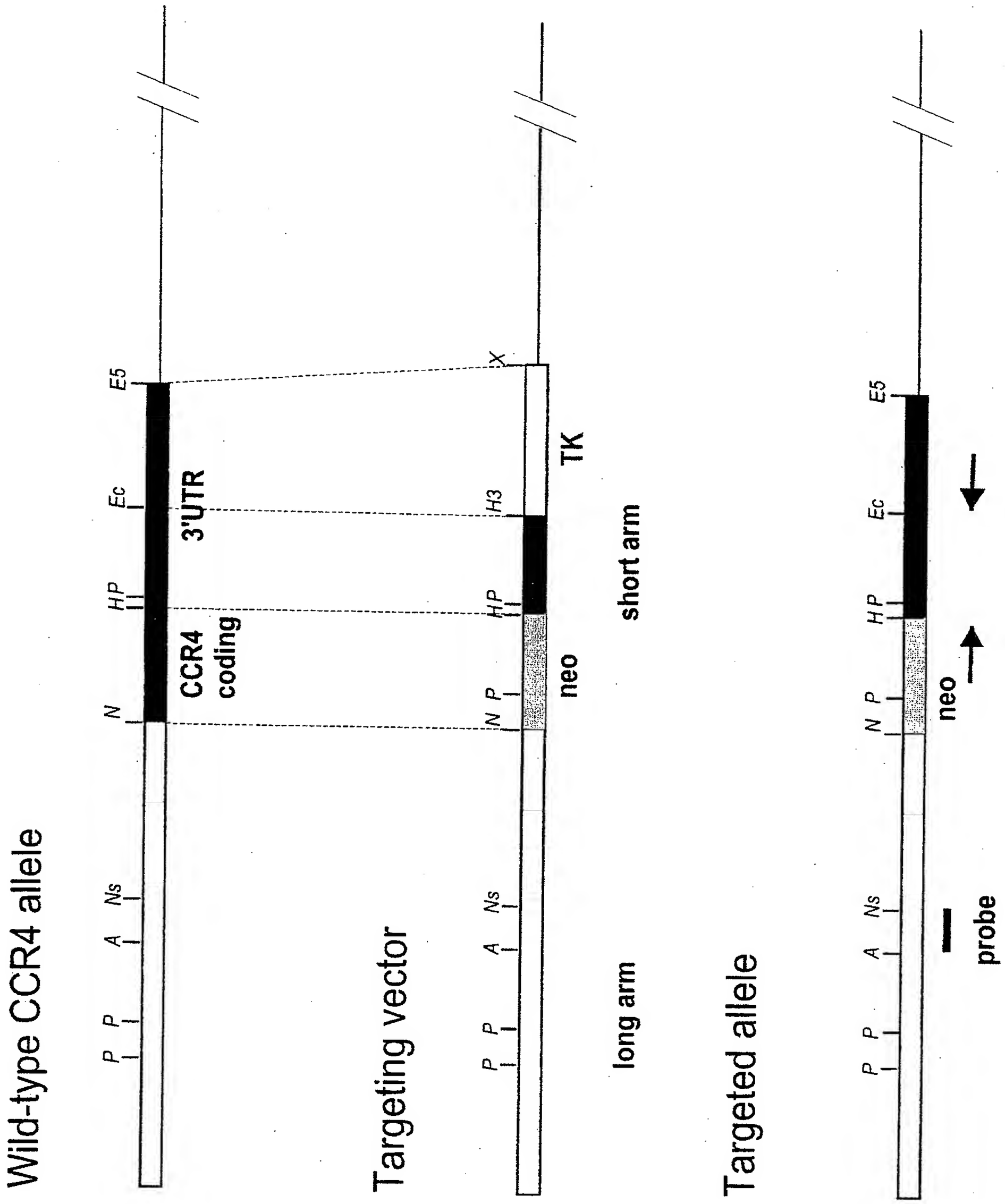


Fig. 1a

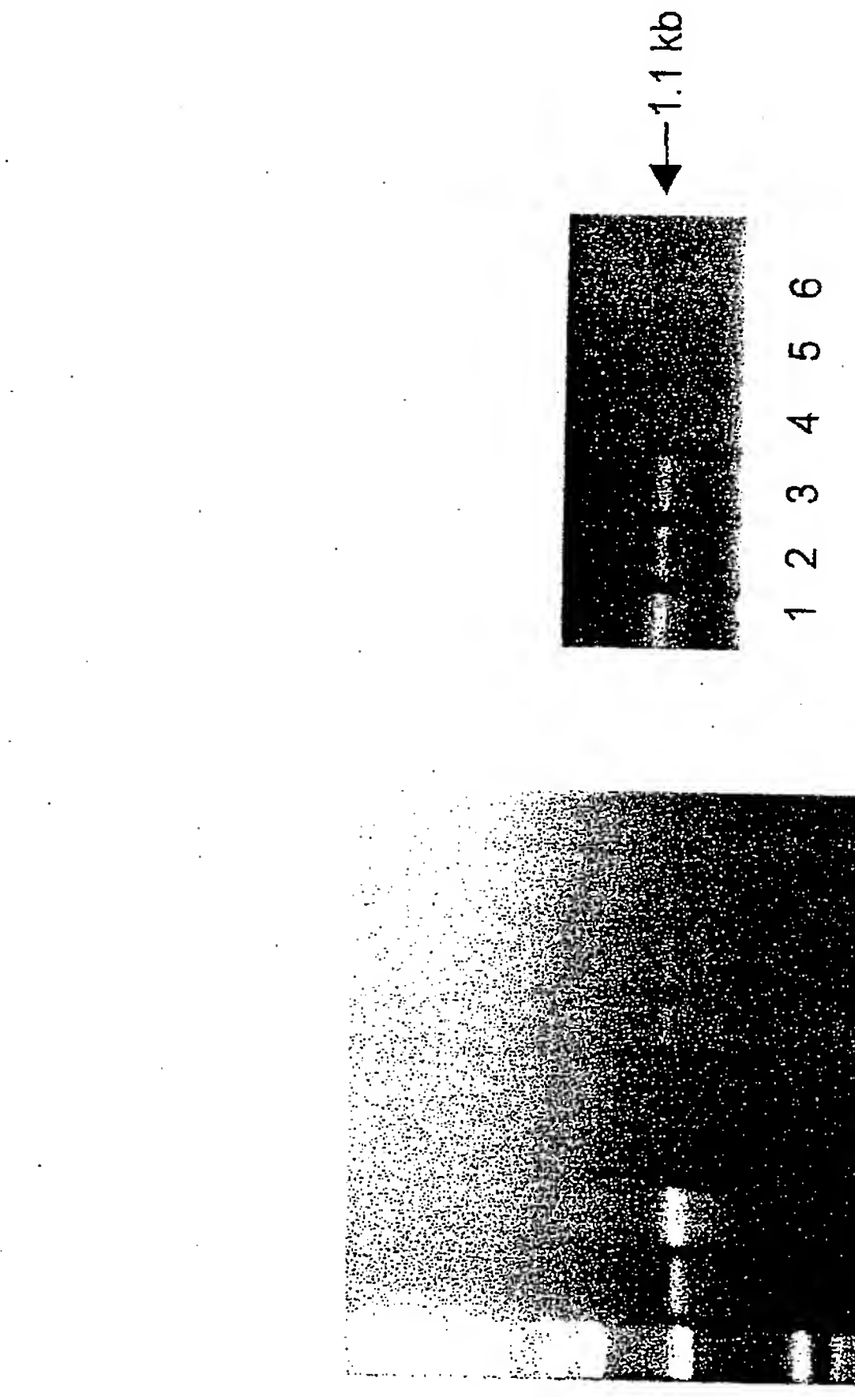


Fig. 1d

Fig. 1c

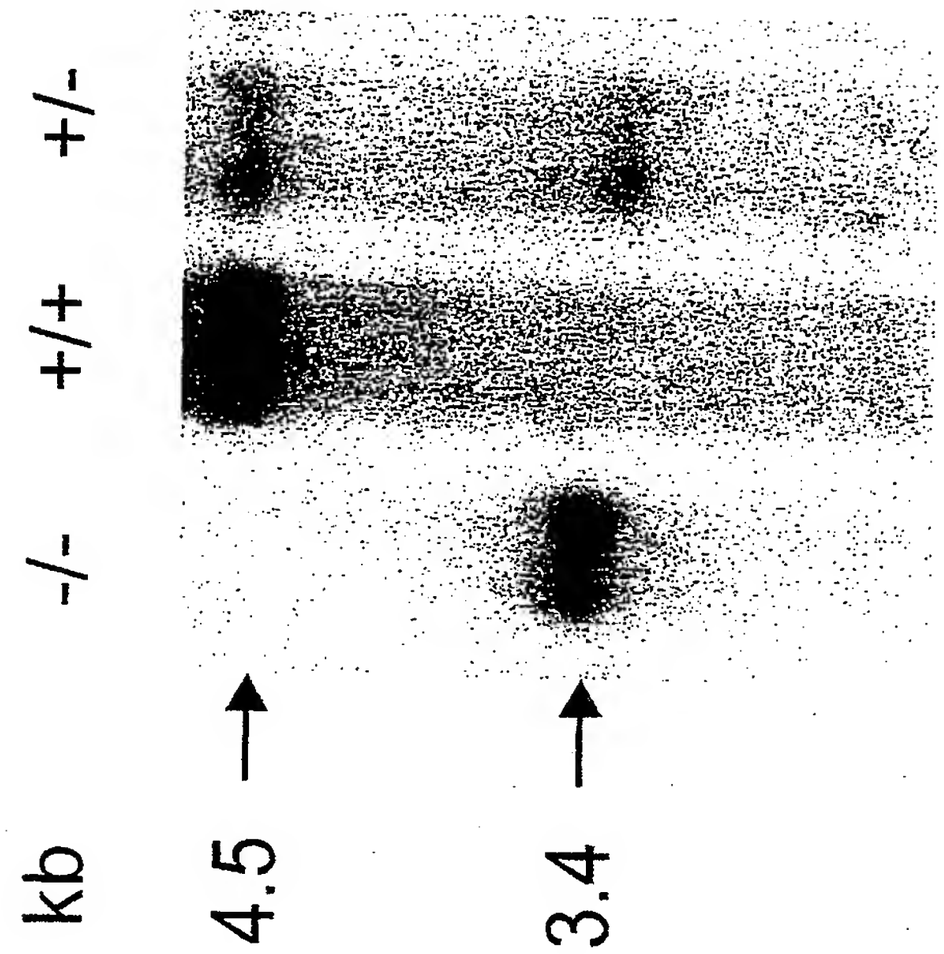


Fig. 1b

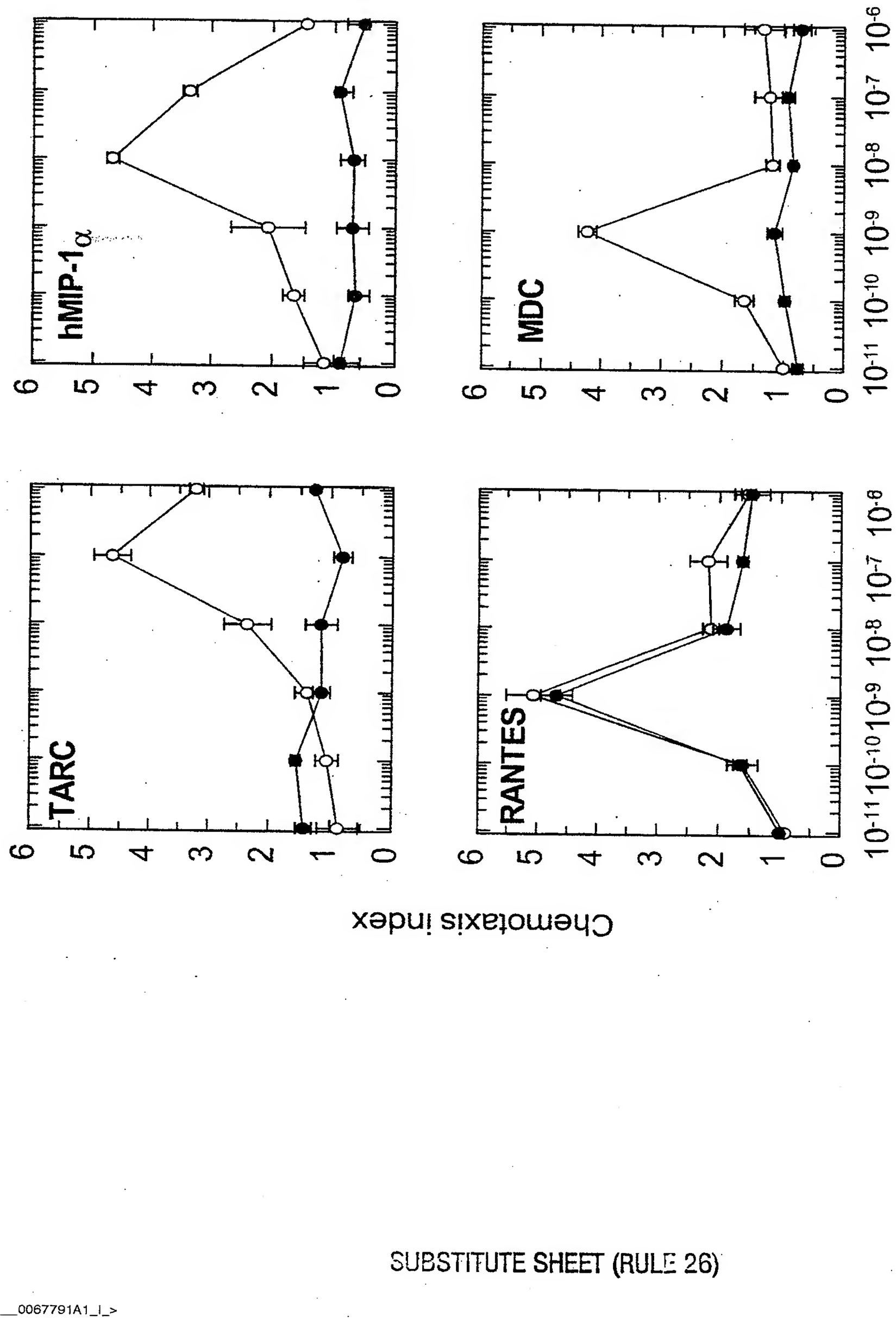


Fig. 1e

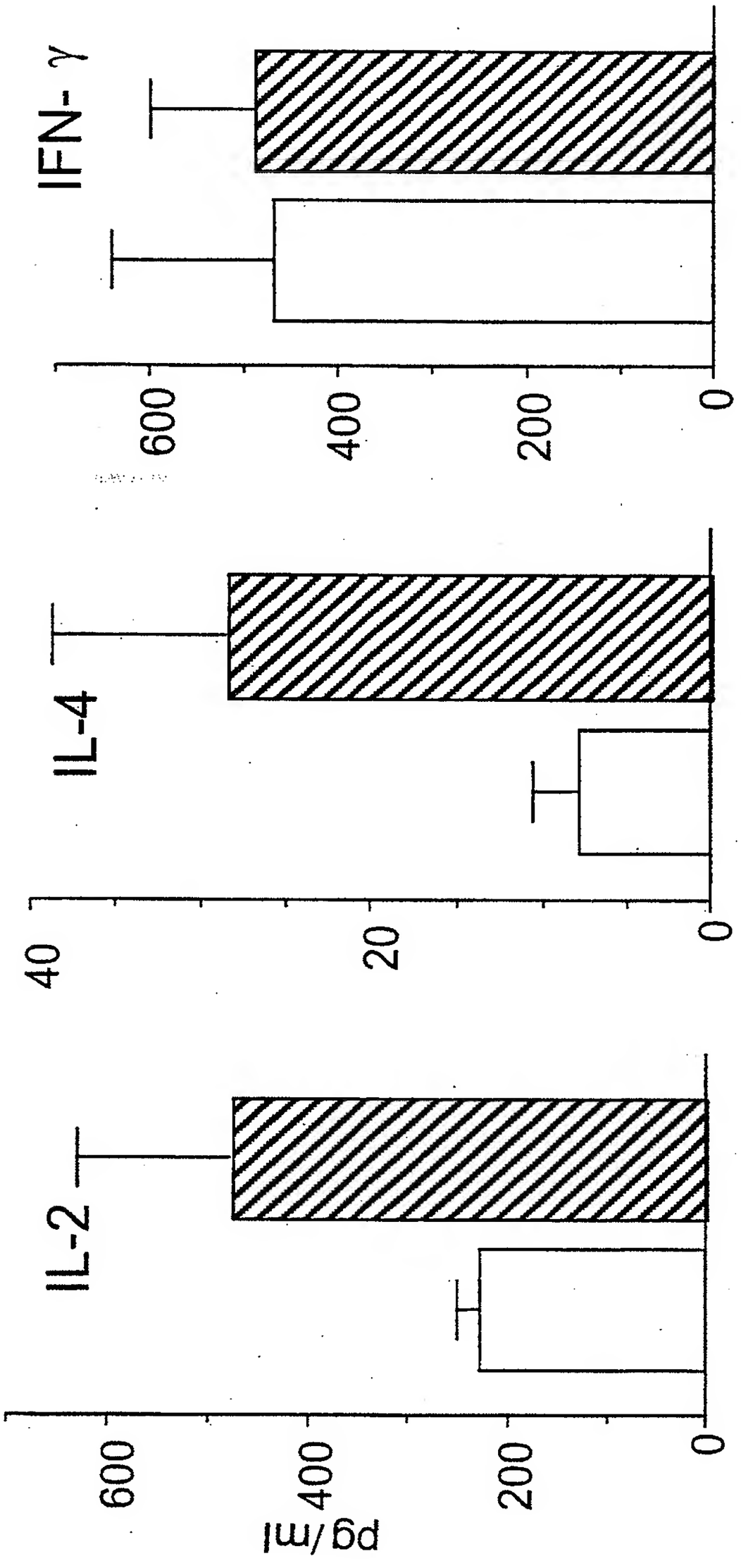


Fig. 2a

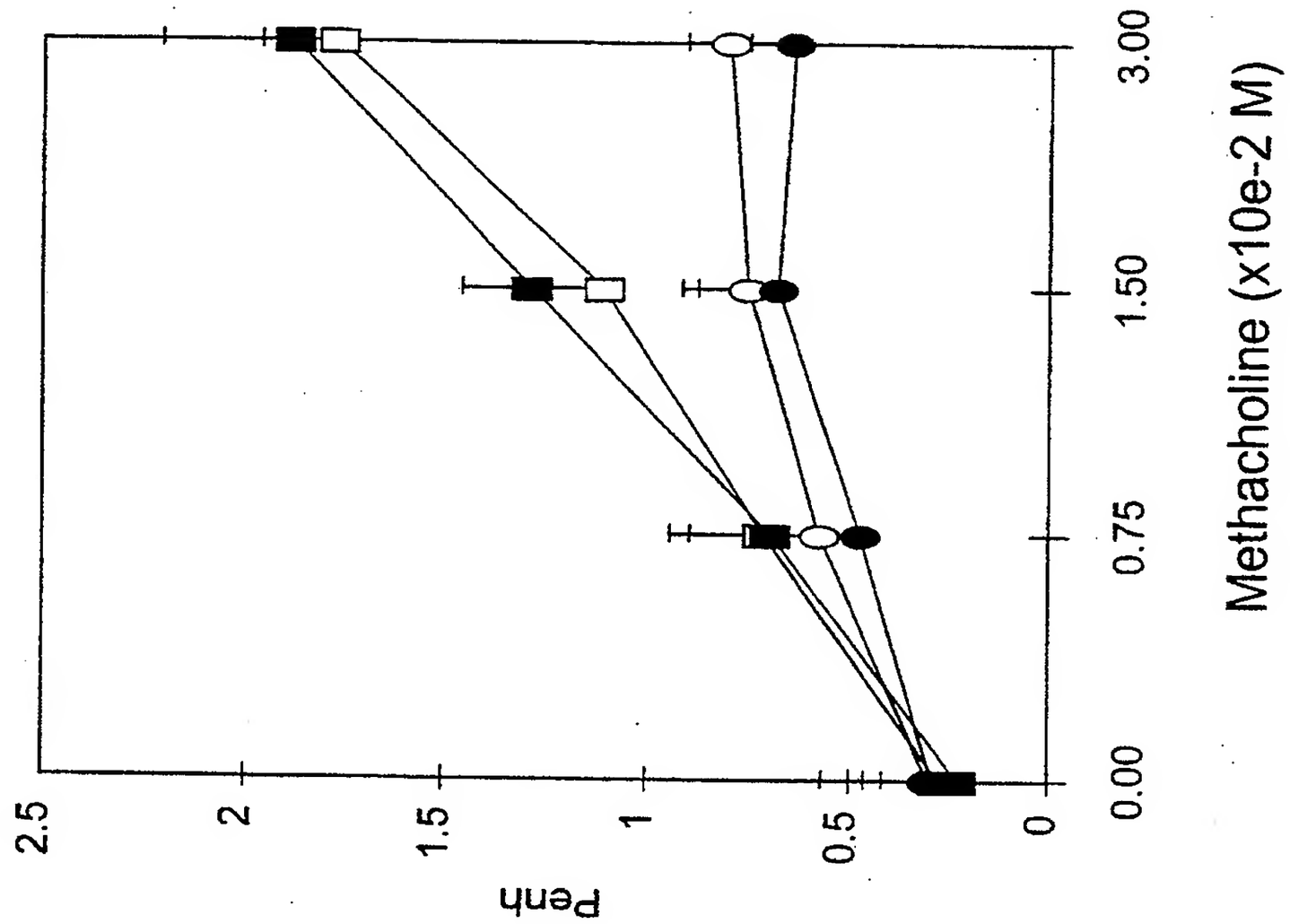


Fig. 2c

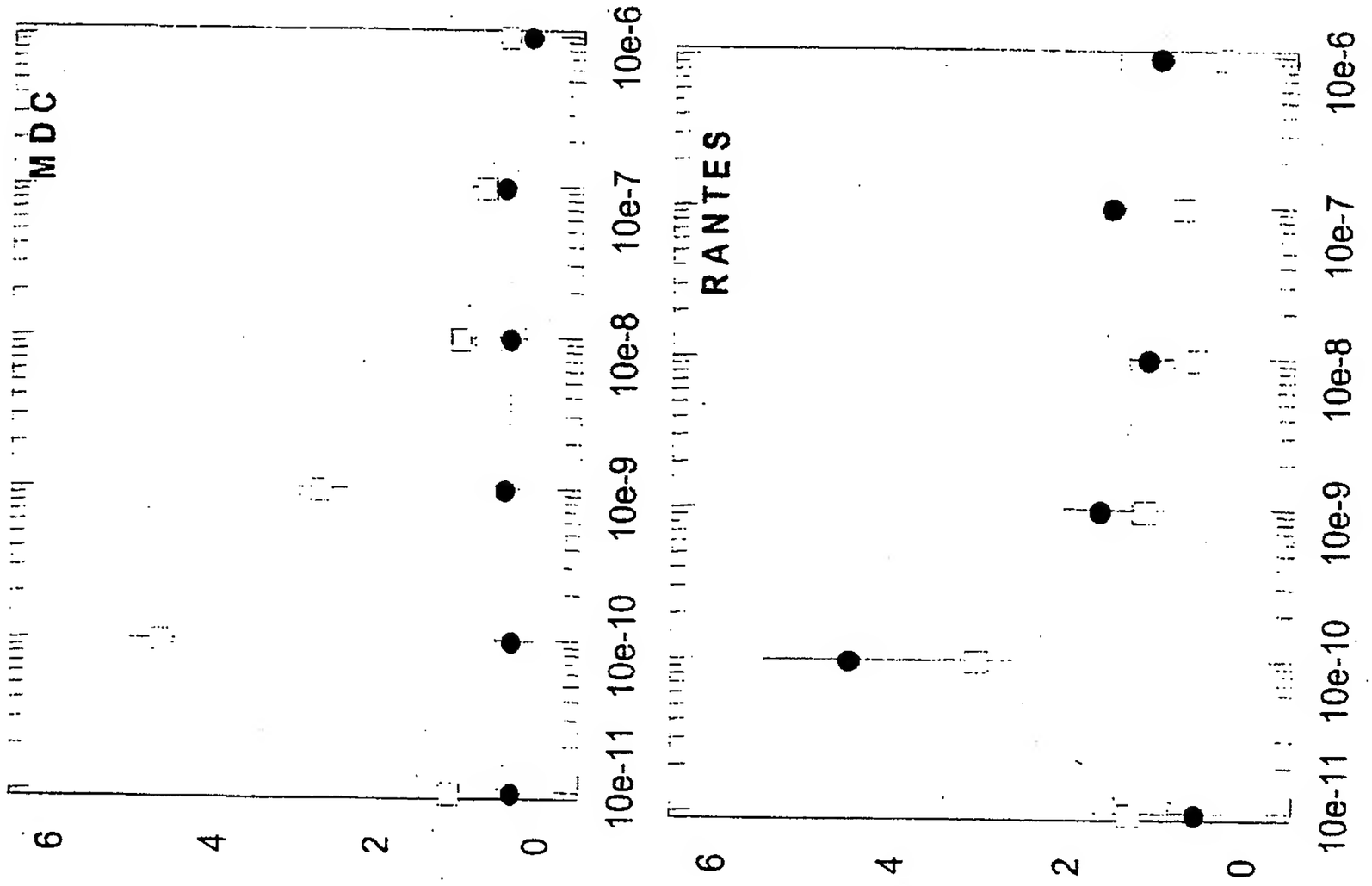


Fig. 2b

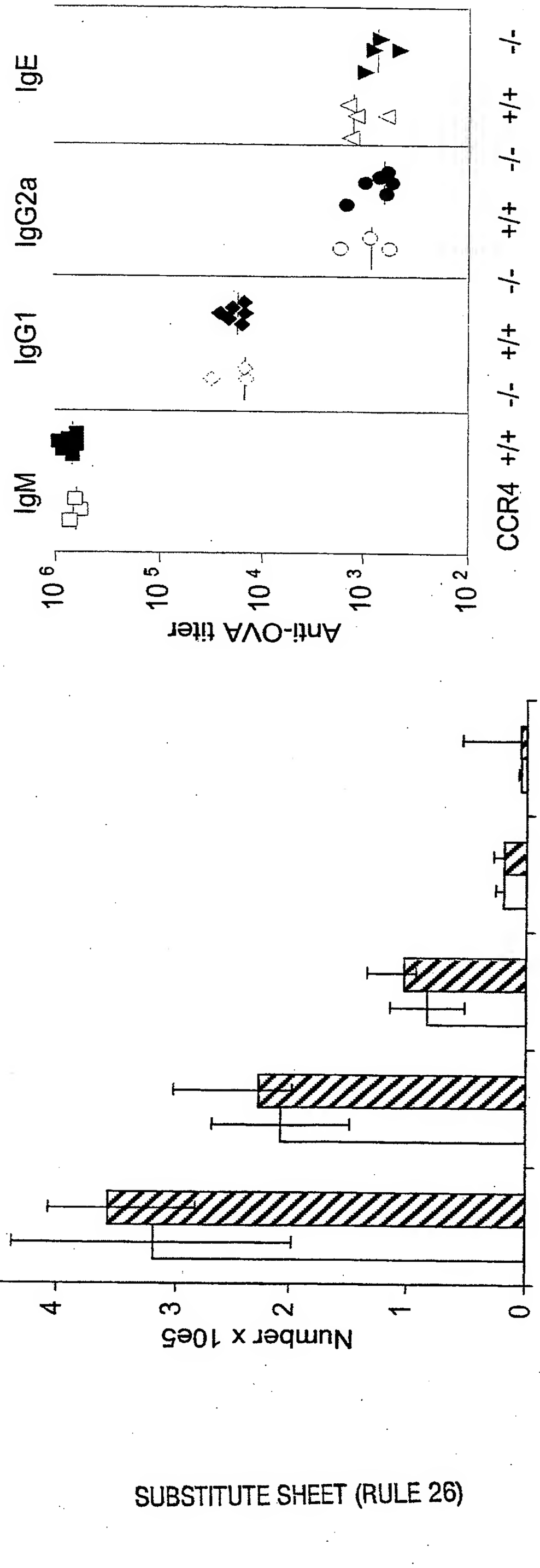
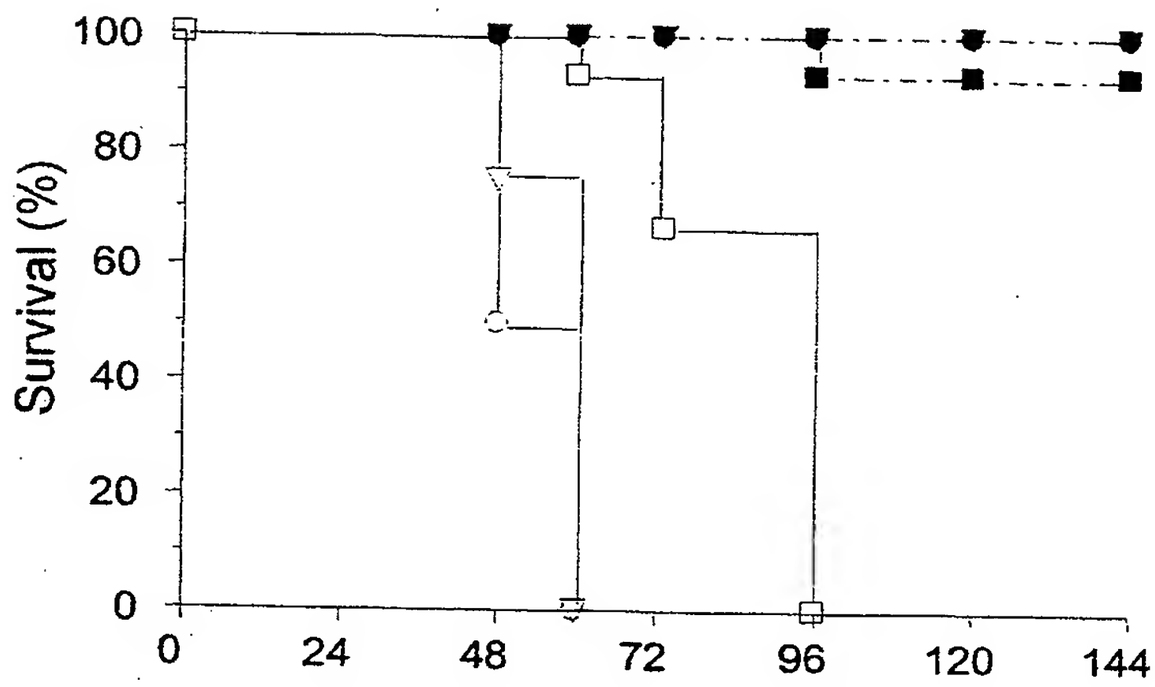


Fig. 2e

Fig. 2 d



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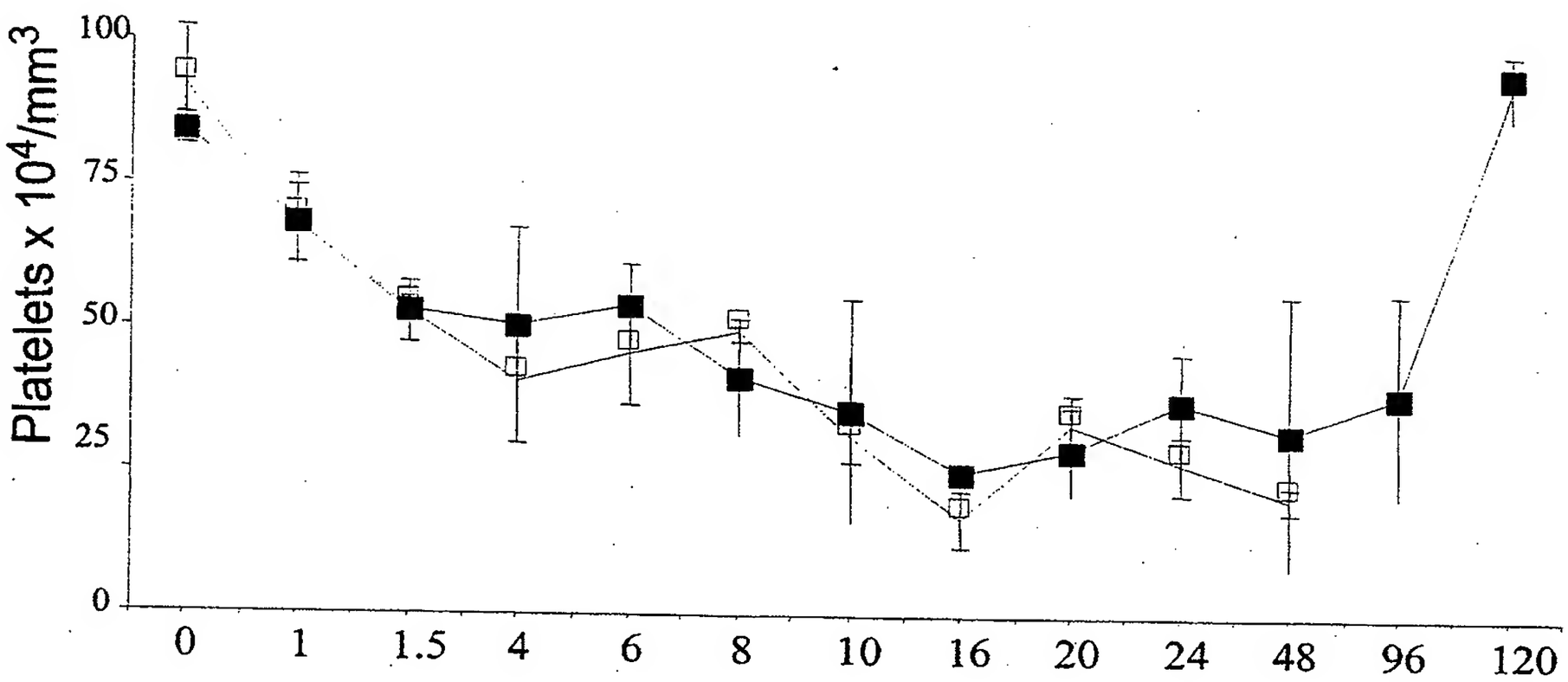

Fig. 3b

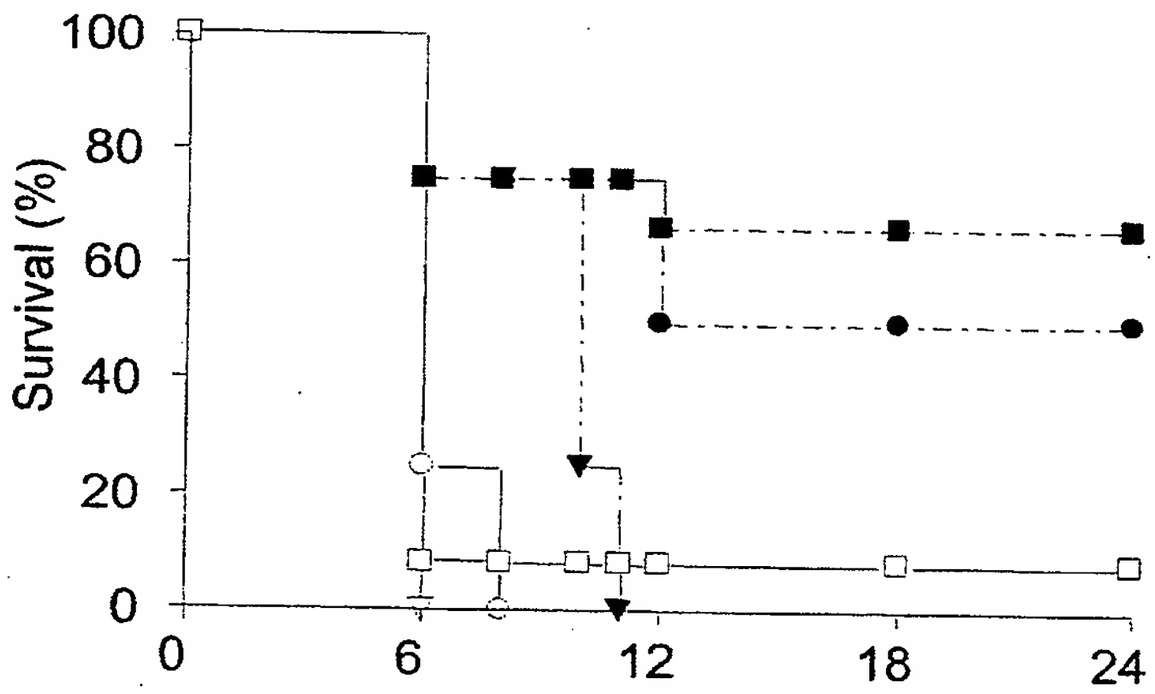

Fig. 3c

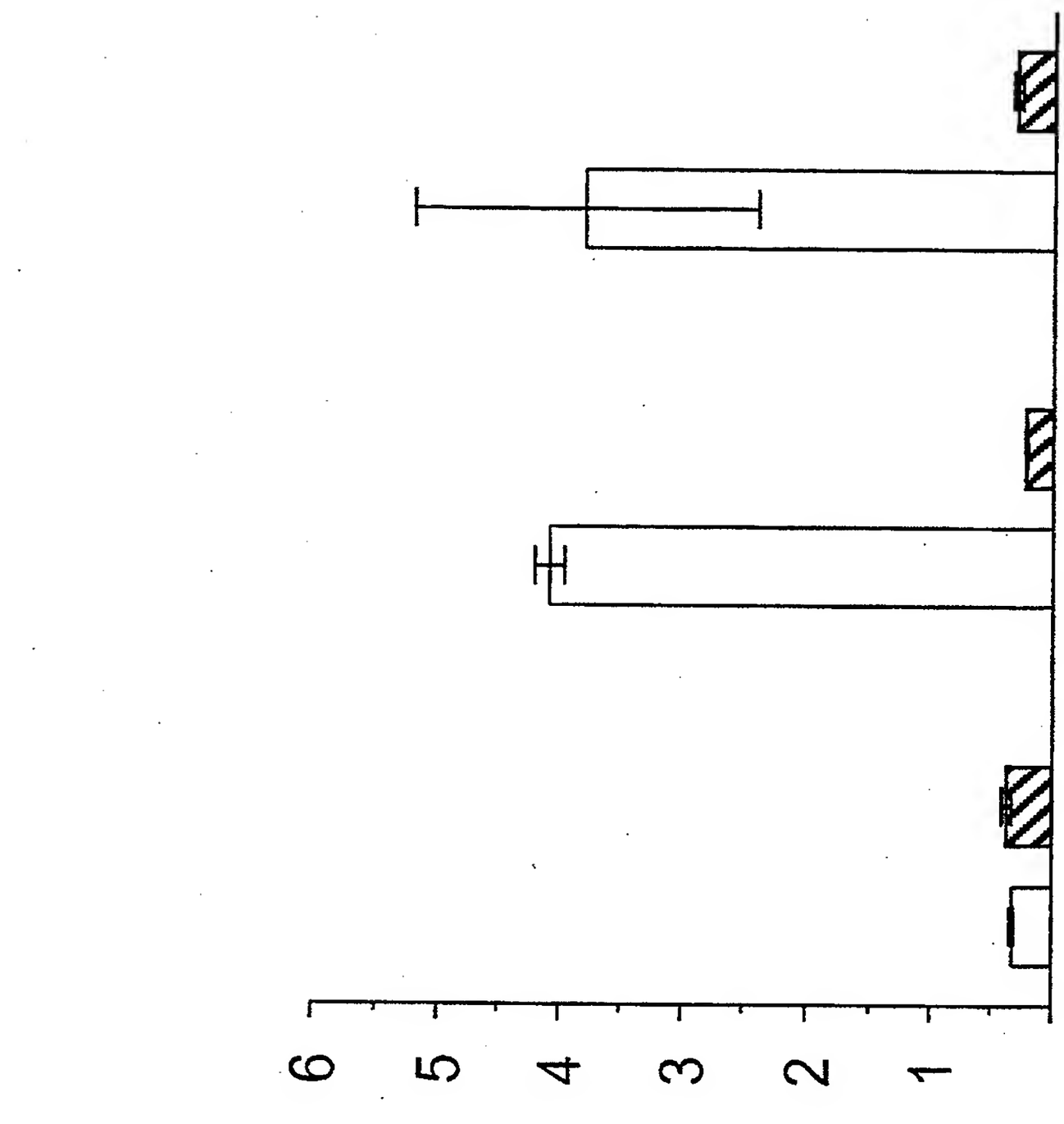

Fig. 3d

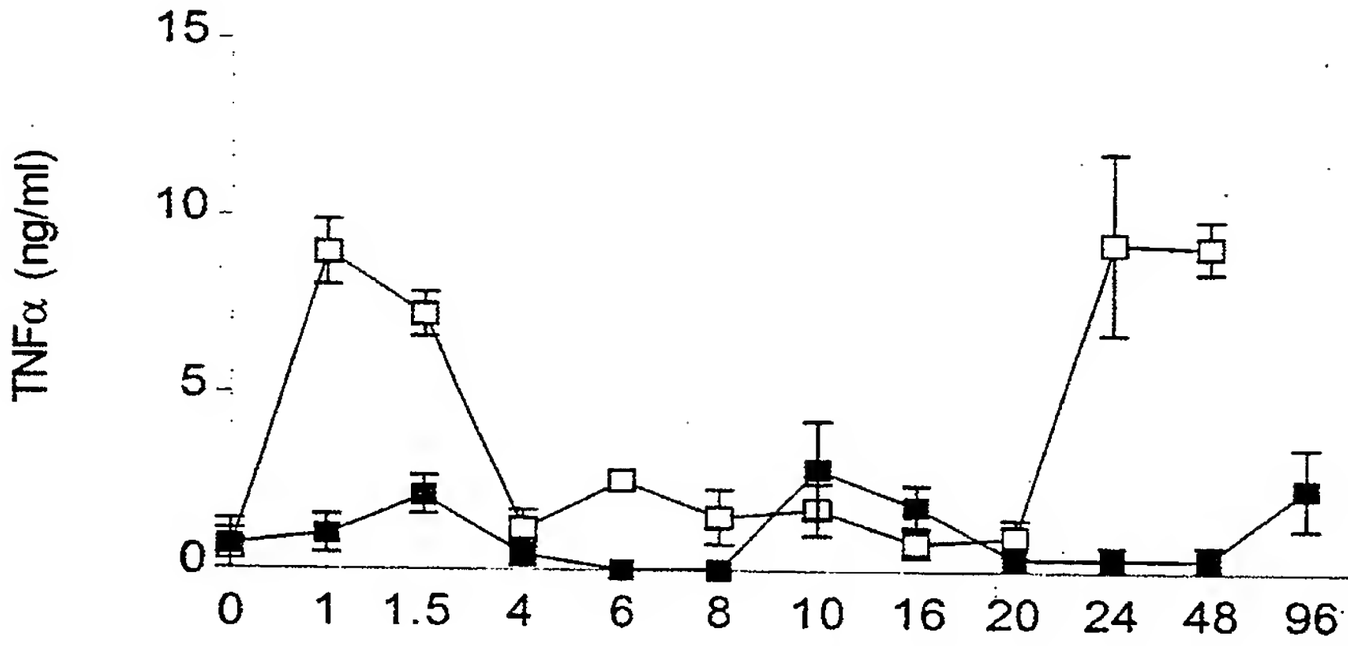

Fig. 4a

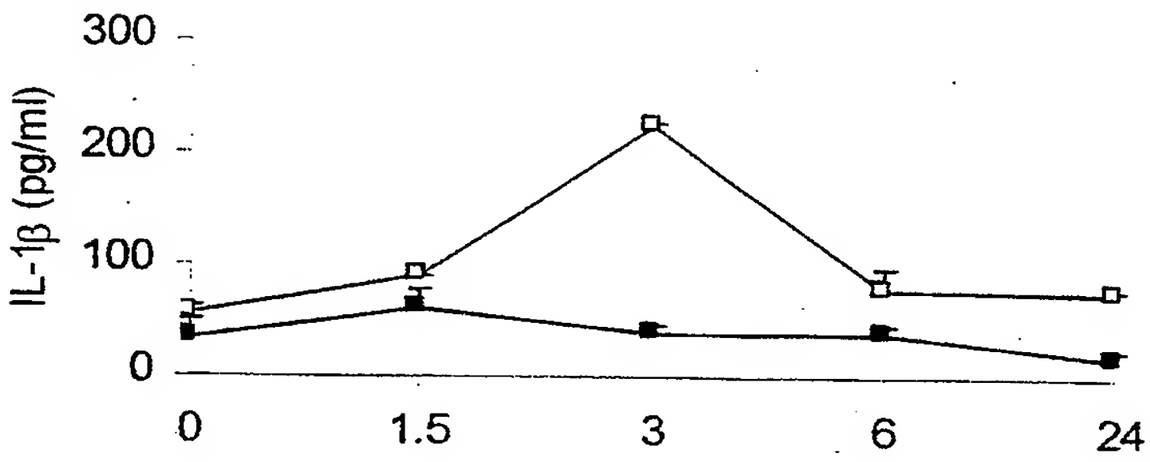

Fig. 4b

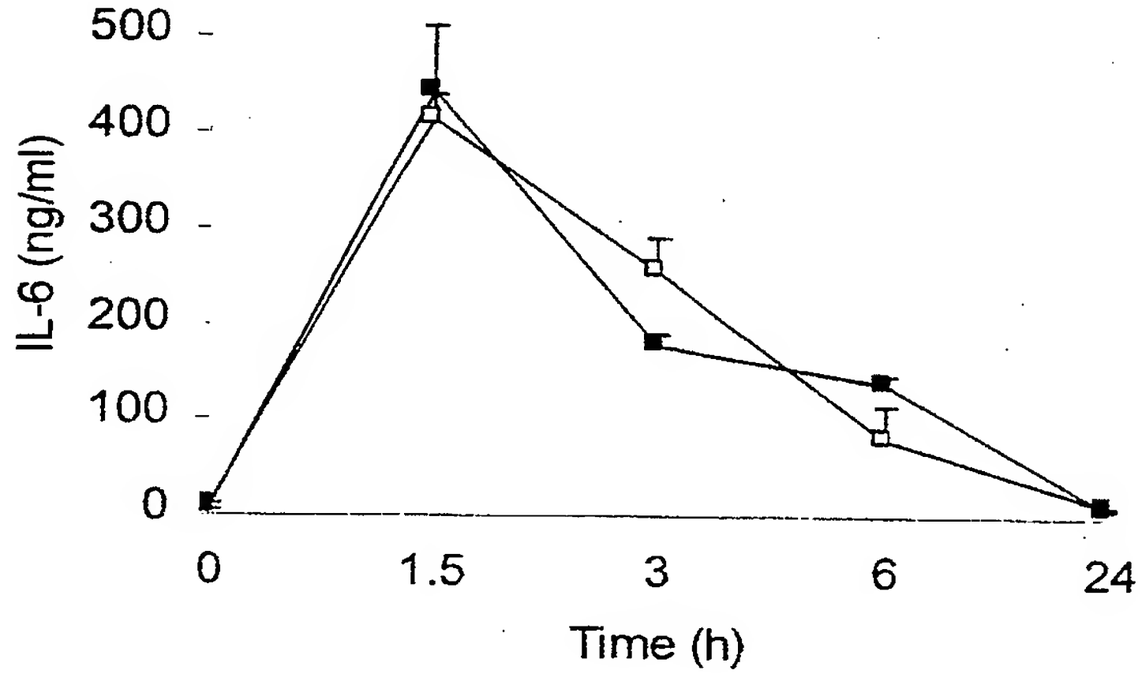

Fig. 4c

Fig. 5a

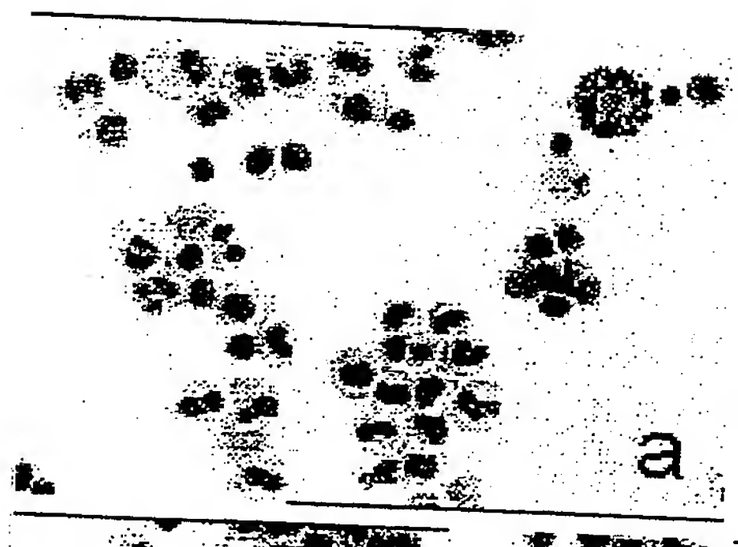

Fig. 5b

Fig. 5c

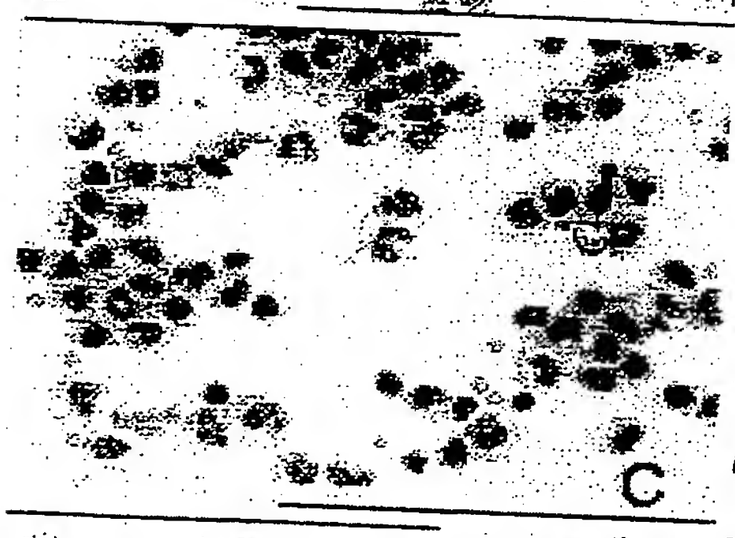

Fig. 5d

Fig. 5e

Fig. 5f

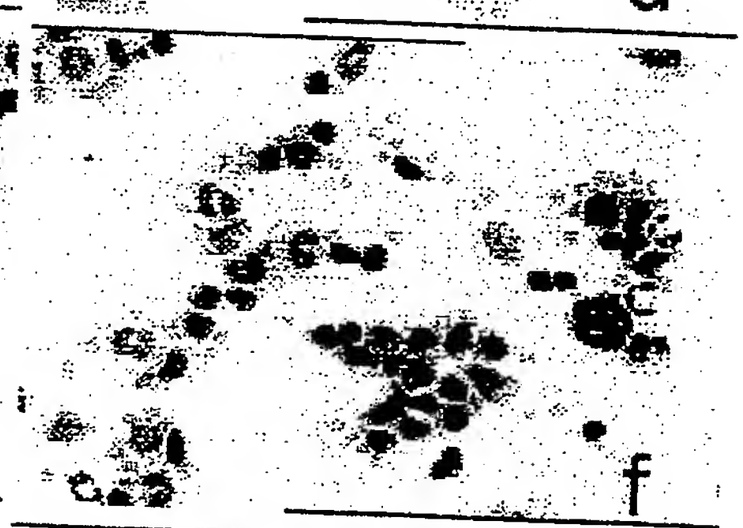

Fig. 5g

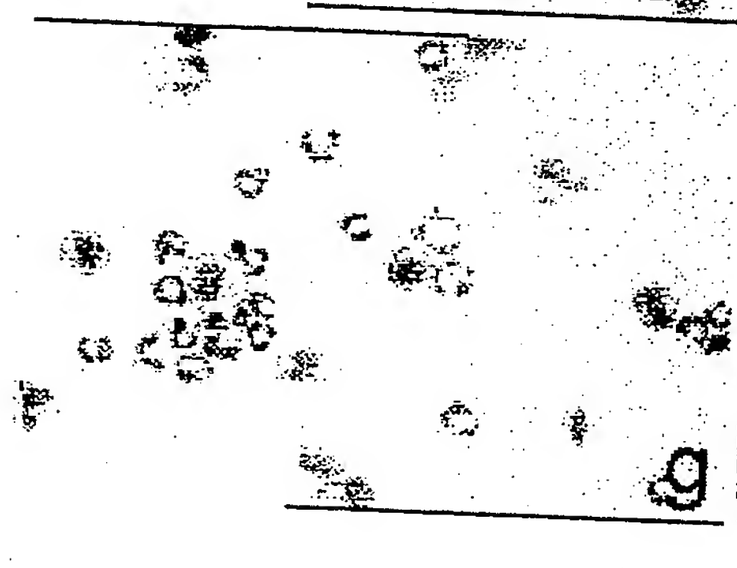

Fig. 5h

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+/+

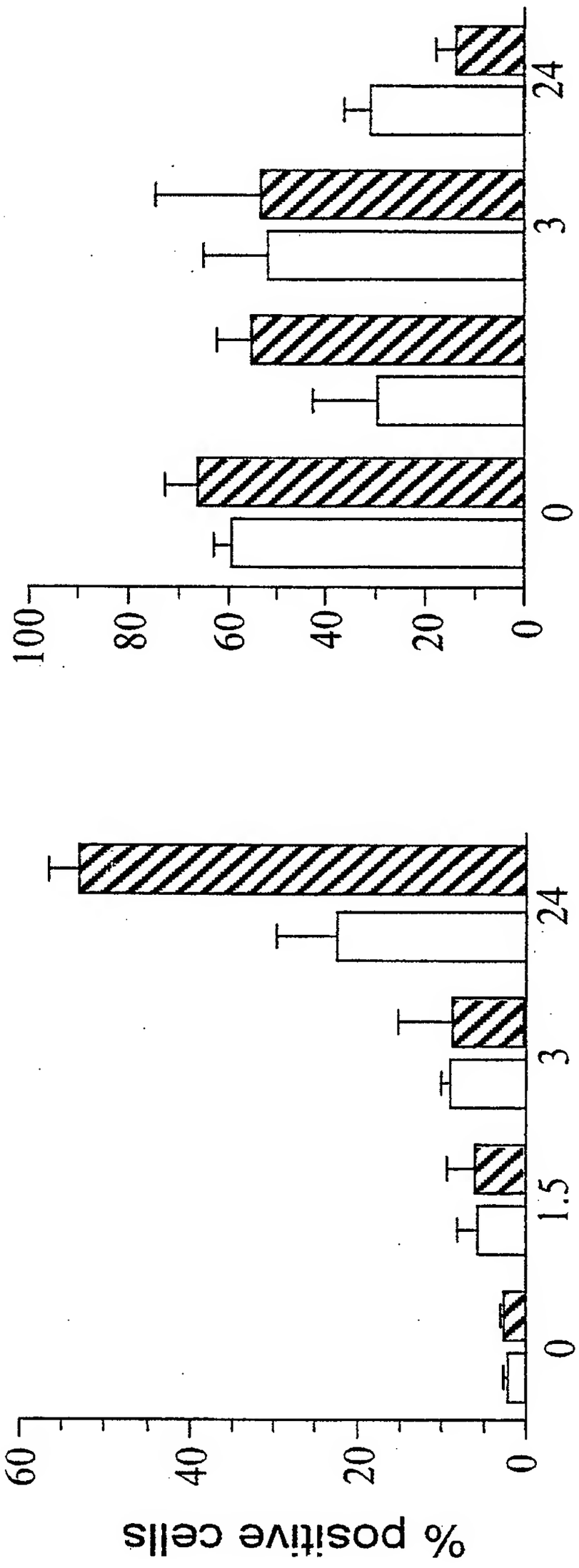

Fig. 6a

Fig. 6b

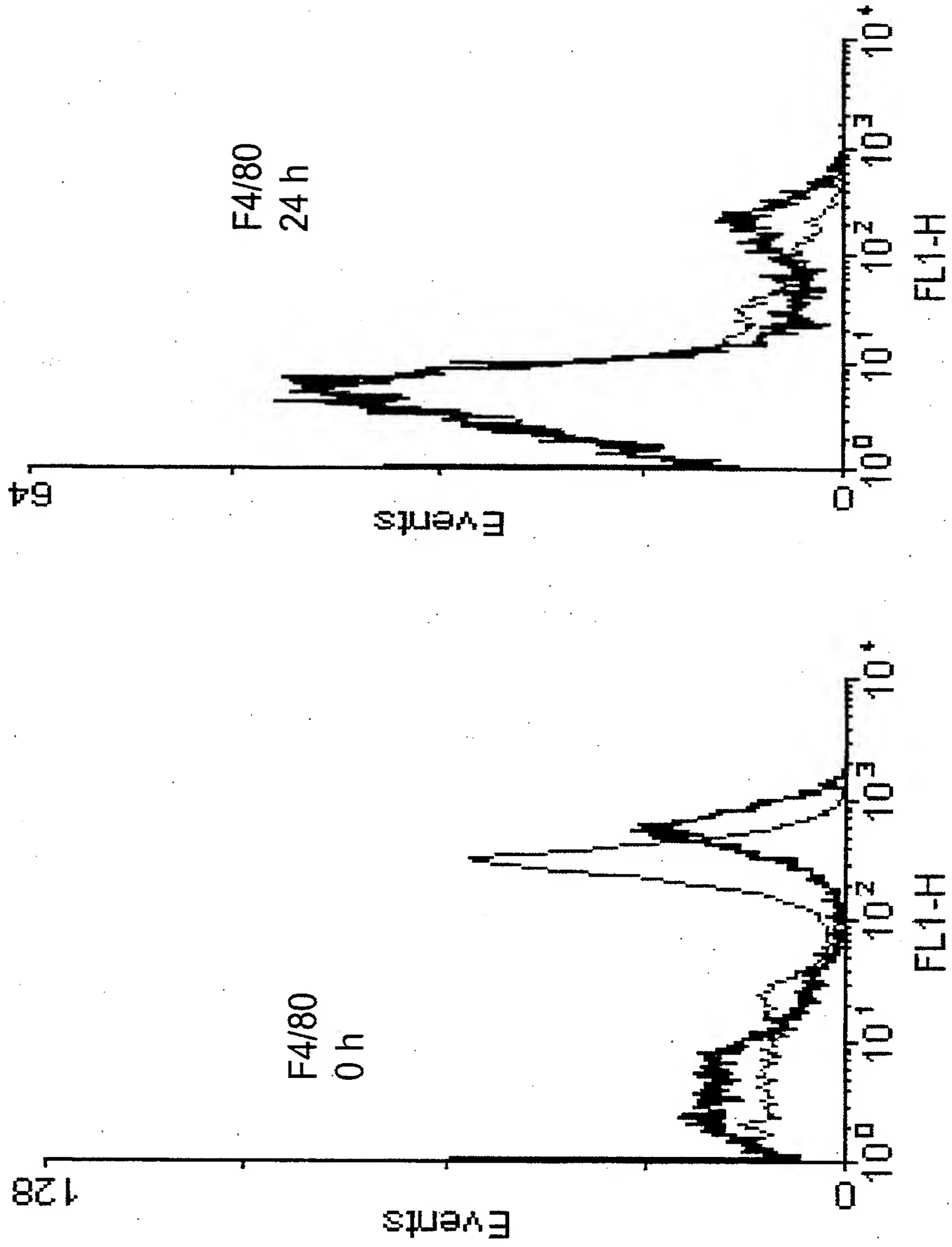

Fig. 6d

Fig. 6c

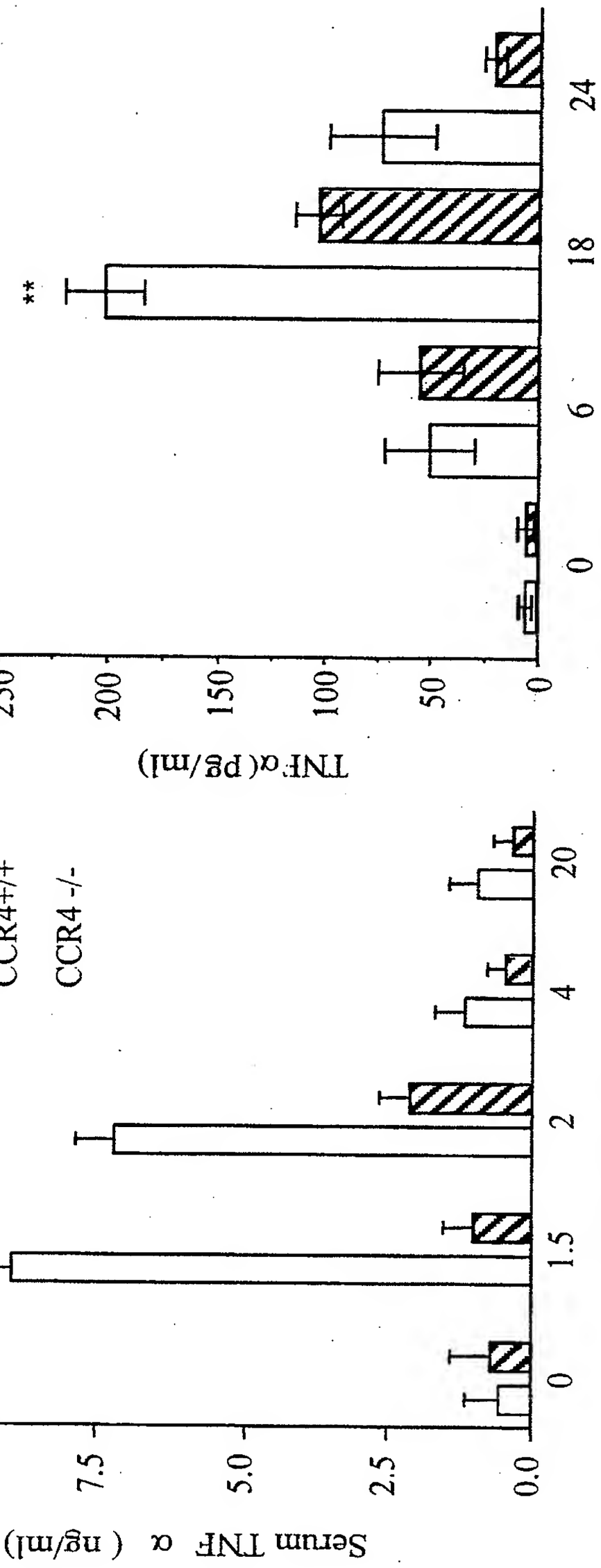

Fig. 7b

Fig. 7a

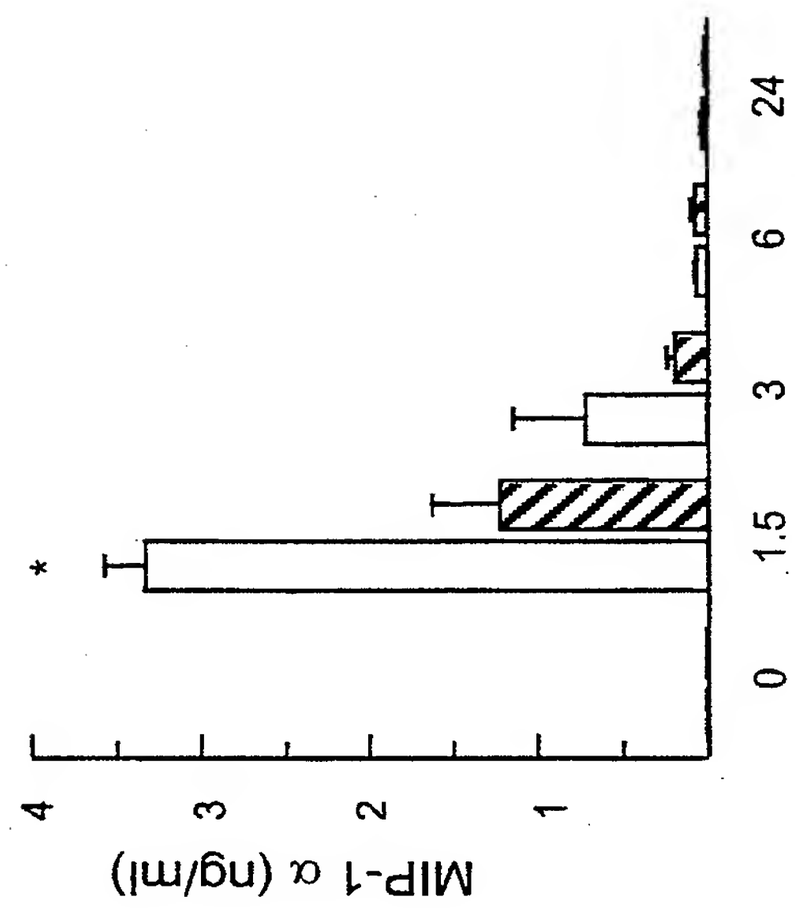

Fig. 7d

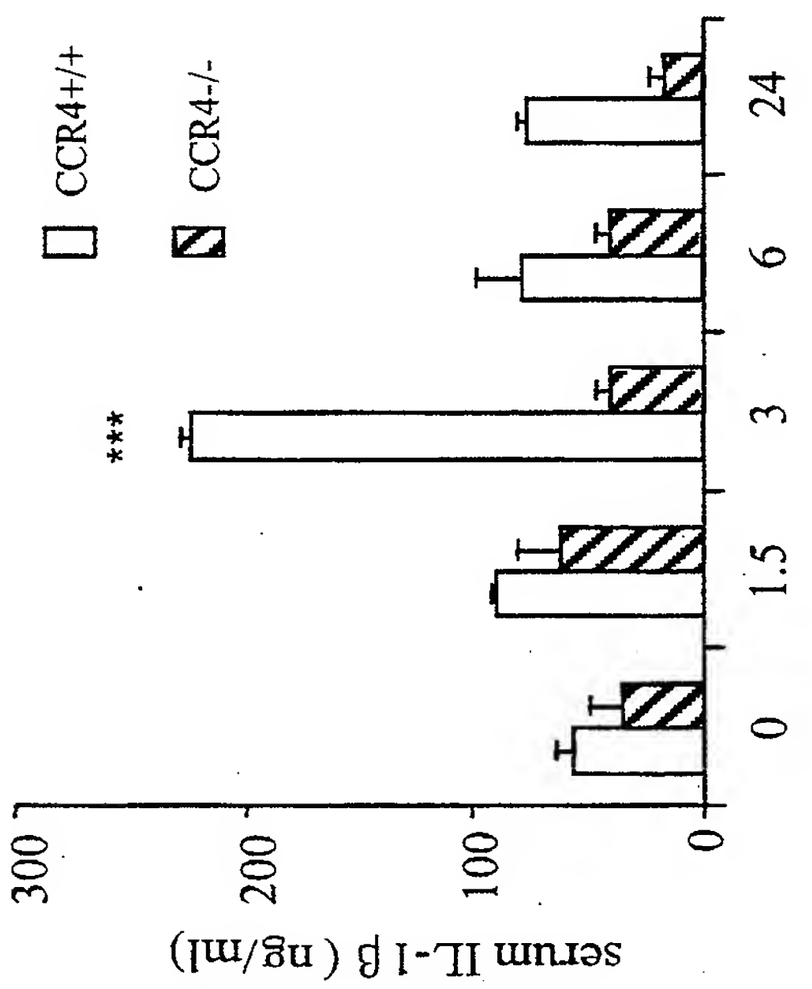

Fig. 7c

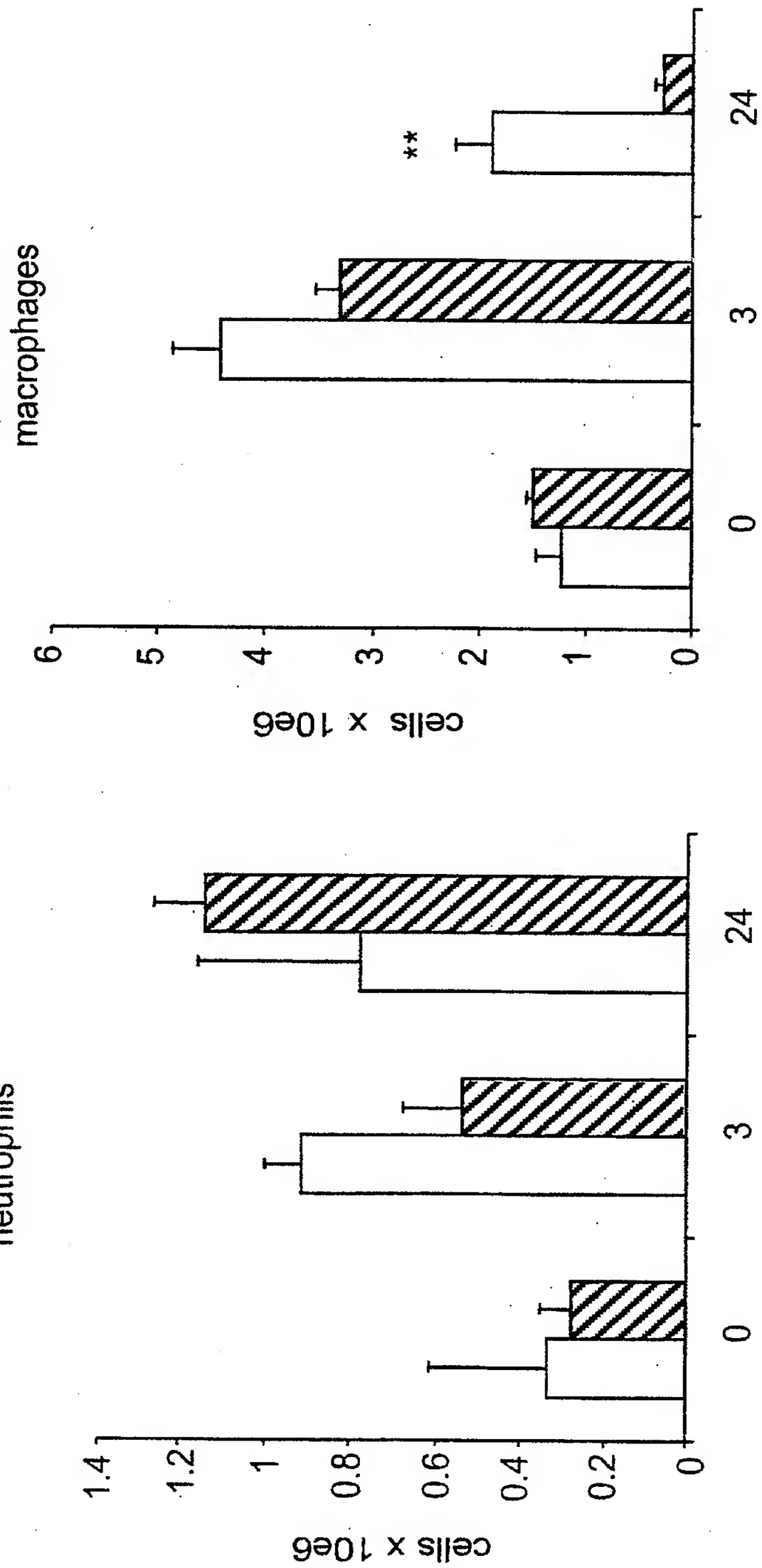

Fig. 8a

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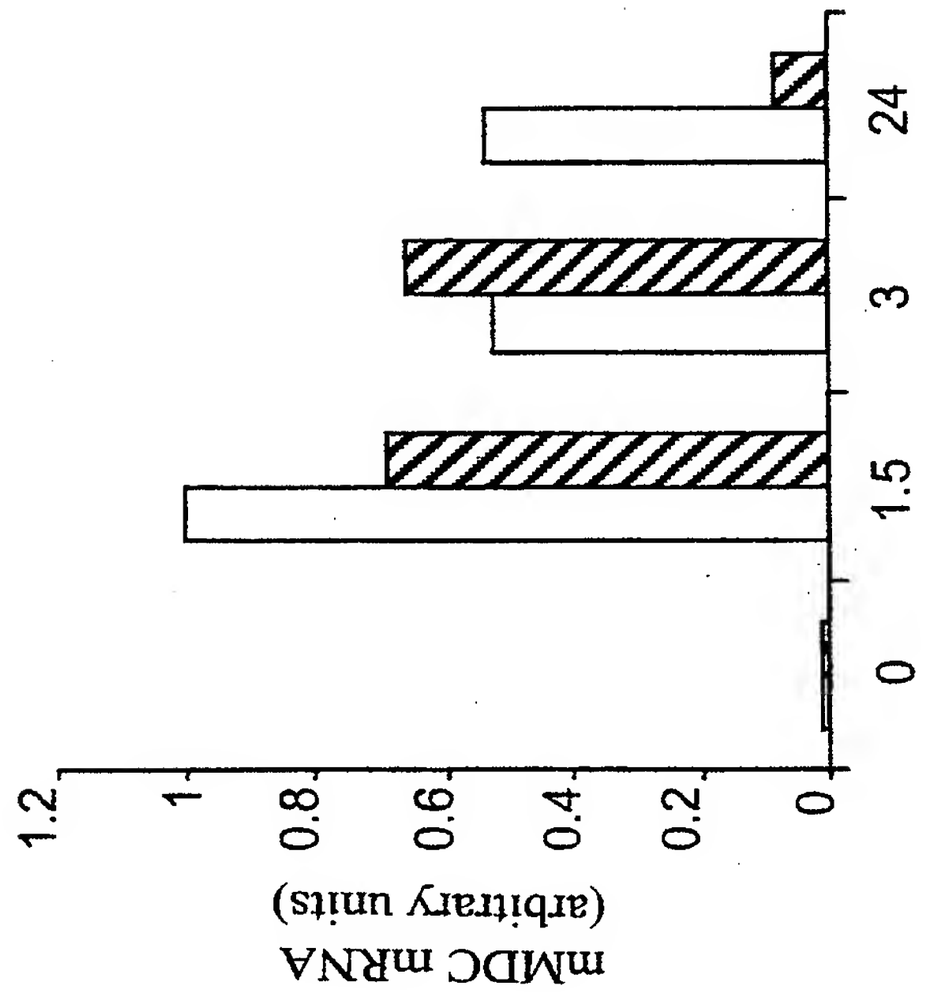

Fig. 8d

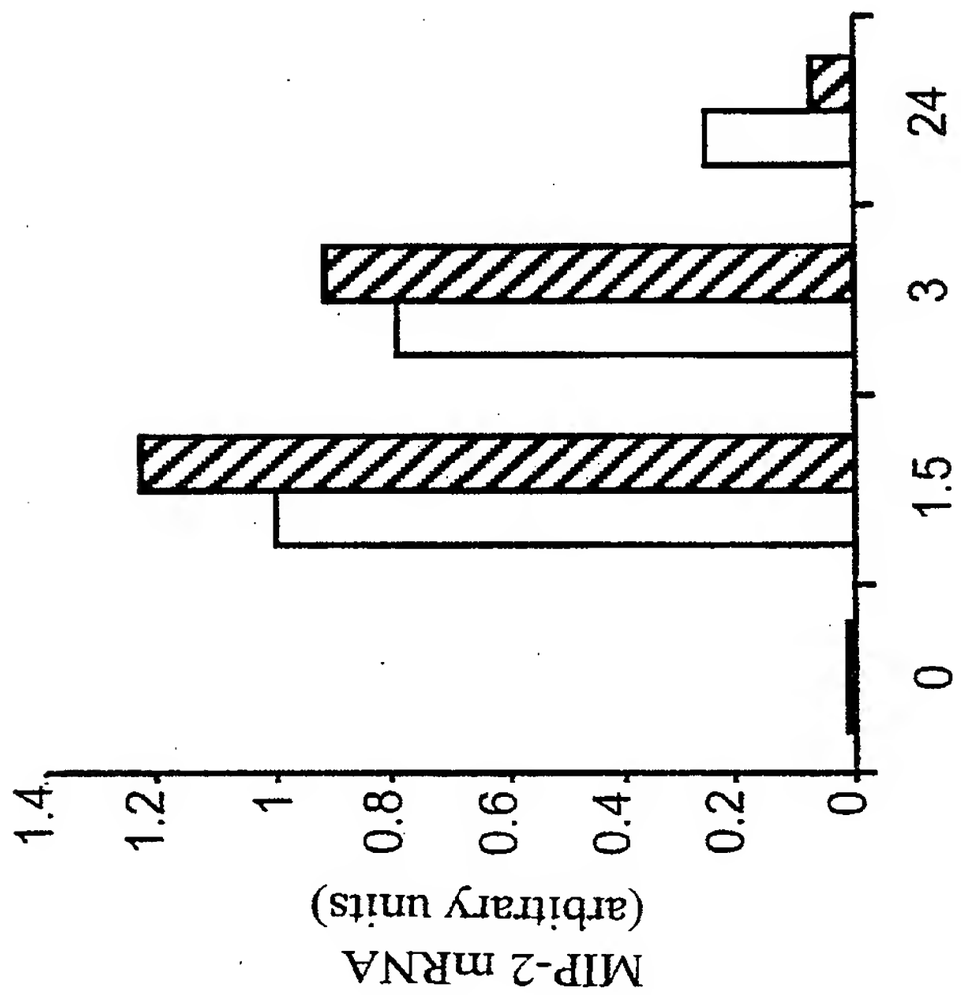

Fig. 8c

INTERNATIONAL SEARCH REPORT

national Application No
PCT/EP 00/04018

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K39/395 A61P31/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
BIOSIS, EMBASE, WPI Data, PAJ, EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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X	WO 99 04794 A (MERCK & CO. LTD.) 4 February 1999 (1999-02-04) page 99, line 3 - line 7 page 101, line 23 - line 24 claims	2,3,7,9, 10
X	WO 98 27815 A (MERCK & CO. INC.) 2 July 1998 (1998-07-02) page 14, line 32 -page 15, line 2 page 17, line 29 - line 30 claims	2,3,7,9, 10

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
12 July 2000	19/07/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Nooij, F

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INTERNATIONAL SEARCH REPORT

national Application No
PCT/EP 00/04018

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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A	J. DURIG ET AL.: "Expression of macrophage inflammatory protein-1alpha receptors in human CD34+ hematopoietic cells and their modulation by tumor necrosis factor-alpha and interferon-gamma." BLOOD, vol. 92, no. 9, 1 November 1998 (1998-11-01), pages 3073-3081, XP000867033 NEW YORK, NY, USA page 3075, left-hand column, line 28 -page 3077, left-hand column, line 22	1-10
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A	H. YONEYAMA ET AL.: "Pivotal role of TARC, a CC chemokine, in bacteria-induced fulminant hepatic failure in mice." JOURNAL OF CLINICAL INVESTIGATION, vol. 102, no. 11, 1 December 1998 (1998-12-01), pages 1933-1941, XP000867034 NEW YORK, NY, USA discussion abstract	1,2,6-10
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Information on patent family members

national Application No
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